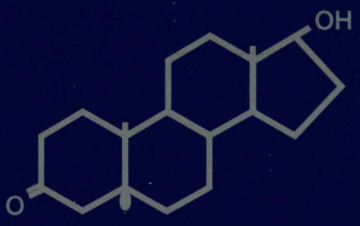
steroid 5α-REDUCTASE ISOZYME ACTIVITIES

in rat and human androgen target tissues



Paul N. Span

steroid

5α-REDUCTASE ISOZYME ACTIVITIES

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Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

Proefschrift ter verkrijging van de graad van doctor aan de Katholieke Universiteit Nijmegen, volgens besluit van het College van Decanen in het openbaar te verdedigen op maandag 22 januari 1996 des namiddags te 3.30 uur precies

door

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geboren op 25 april 1967 te Utrecht

Druk: Quickprint Nijmegen

Nijmegen

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PAUL N. SPAN

opgedragen aan mijn ouders,
ter nagedachtenis aan mijn moeder.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Span, Paulus Nicolaas

Steroid 5α-reductase isozyme activities in rat and human androgen target tissues / Paulus Nicolaas Span. - [S.l. : s.n.] (Nijmegen : Quickprint Nijmegen). - Ill.
Thesis Katholieke Universiteit Nijmegen. - With ref. - With summary in Dutch.
ISBN 90-9008912-8
Subject headings: 5α-reductase / steriod metabolism / prostatic hyperplasia.

CONTENTS

page:

CHAPTER 1
INTRODUCTION

1.1 PREFACE

Isozymes of 5α-reductase (E.C. 1.3.99.5) mediate in the wide variety of actions elicited by testosterone in androgen-target cells, and play a role in the catabolism and excretion of steroids in non-androgen target cells. Recent progress in the research on 5α-reductase with current molecular biological techniques has elucidated many of the discrepancies in earlier reports on enzymatic activity measurements of 5α-reductase in rat and human tissues. However, our apprehension of the *in vivo* function of 5α-reductase in normal and diseased tissue is still clouded by kinetic peculiarities of the isozymes. As the two now established isozymes catalyze the same reaction, quantification of their activities is difficult, even more so due to differences in opinion as to the best method of quantification.

The introductory chapter addresses the crucial roles of 5α-reduction in the effectuation and regulation of testosterone action (*section 1.2*), and the role of isozymes of 5α-reductase herein (*section 1.3*). In the ensuing sections, the literature on 5α-reductase, from gene structure (*section 1.4*) to protein structure (*section 1.5*) and to enzymatic activity (*section 1.6*) is discussed. Furthermore, data on the tissue-specific, cell-type-specific, and intracellular localization of the isozymes are presented (*section 1.7*). *Section 1.8* presents the scope of this thesis.

1.2 ROLE OF 5α-REDUCTASE IN ANDROGEN ACTION

The constituting parts of all higher animal organisms (cells, tissues, organs) are integrated by a complex network of neuronal and endocrine signals. The complexity of this network can be illustrated by the wide variety of effects elicited by the most common male endocrine signal, the steroid hormone *testosterone*.

This steroid is the principal androgen in the human male and is pivotal in all stages of development, from the embryo, foetus and neonate to the sexually mature adult [128]. It is secreted by the testes and circulates in the bloodstream, either free or bound to specific *binding proteins* [223, 224]. In particular, the non-protein bound fraction of

steroids (approximately 1-2% in the case of testosterone) is thought to elicit effects on the different cells of the body (*free hormone hypothesis*) [171]. From the bloodstream, testosterone can ubiquitously diffuse across plasma cell membranes. Remarkably, the effects of this very hormone range from development of the urogenital tract, male type hair-growth, deepening of the voice, increase in musculature to male behaviour and sexual attraction. Furthermore, it exerts mitogenic and haematogenic effects [138].

An important element in providing the desired specificity for the indiscriminately distributed testosterone is the *androgen receptor* [23], member of the steroid/thyroid hormone/retinoic acid receptor family [158]. Cells in an organism that display this receptor are able to interact with testosterone. After binding, the hormone-steroid receptor complex binds to hormone response elements in the DNA of the cell, resulting in the switching on, or off, of the appropriate genes within that specific cell-type [159]. However, the androgen receptor can be found in almost every organ in the human body, with the notable exception of the spleen [123]. In the disorder of androgen insensitivity syndrome (AIS), there is a complete lack of, or structural change in this androgen receptor [168]. Patients with the complete form of AIS, though genetically male (XY genotype), have female external genitalia, a blunt-ended vagina, absent uterus, abdominal or inguinal testes and defective sexual hair growth [18, 45, 63]. In these patients, testosterone has been unable to initiate the development of Wolffian ducts into vasa deferentia, seminal vesicles, ejaculatory ducts and epididymides. Female internal structures, however, are absent because of the activity of Mullerian inhibitory factor produced by the testes. This disorder stipulates the prerequisite of testosterone action in male sexual development and its wide range of possible effects.

Another important factor determining the physiological effects of testosterone is the *local metabolism* of this steroid in the target cells [197]. Two of the major pathways in the metabolism of testosterone are *aromatization* and *5α-reduction*, resulting in the formation of estrogens and dihydrotestosterone (DHT) respectively. For instance, the effect of testosterone on male sexual behaviour and sexual orientation is mediated by its aromatization to 17ß-estradiol in specific parts of the central nervous system, and subsequent binding to the estrogen receptor [138, 144]. The effect of testosterone on male pattern hair growth results from its 5α-reduction to DHT in the hair follicles [166]. Remarkably, testosterone and DHT bind to the same (androgen) receptor. The mechanism by which one receptor can differentiate between these two ligands is still

not known. Testosterone does have a 3-fold lower affinity for the androgen receptor than DHT [209] and high concentrations of testosterone might be able to mimic DHT binding to the androgen receptor [62].

The crucial role of DHT in androgen action has been greatly exemplified by studies of male patients with pseudohermaphroditism who partially or completely lack the enzyme 5α-reductase [48, 229]. The disorder was attributed to the lack of peripheral conversion of testosterone to DHT [87, 220], enabling distinction between the effects of testosterone and DHT per se on androgen target cells. In contrast to patients with the complete form of AIS, male pseudohermaphrodites with 5α-reductase deficiency exhibit normal male internal genitalia derived from Wolffian ducts (vasa deferentia, seminal vesicles, ejaculatory ducts and epididymides) [48, 220, 229]. The androgenic effects of testosterone on these tissues are completed before 5α-reductase is present [192]. At puberty normal male musculature develops, but the external genitalia are ambiguous. The prostate is atrophic [86]. In addition, these patients appear to have less baldness and acne. The normal androgenization of the internal genitalia and male musculature can thus be ascribed to the androgenic action of testosterone, whereas the lack of DHT is responsible for the ambiguity of the external genitalia and atrophy of the prostate. So, both testosterone and DHT have distinctive roles in male sex determination [64].

Thus, if a cell expresses only the androgen receptor and no 5α-reductase, the androgenic effects are elicited by testosterone itself (as is the case in muscle tissue). If a cell does contain 5α-reductase, the androgenic effects of testosterone are mediated by DHT (as in the prostate). Furthermore, if 5α-reductase and other metabolizing enzymes are expressed, the metabolism and subsequent excretion of testosterone is facilitated (as in the liver) (*figure 1.1*). Therefore, androgen target tissues are imparted with the enzymatic machinery for producing the appropriate metabolites of testosterone as (and when) required. In doing so, the cell itself can modify the effect of testosterone.

The enzyme 5α-reductase has been implicated in several diseases and disorders, including benign prostatic hyperplasia (BPH) [191, 228], male pattern baldness [13], hirsutism [95, 106, 186], acne [175] and prostatic cancer [21, 54, 68]. Some of these relationships are, however, disputed [165]. With the isolation of the genes for this

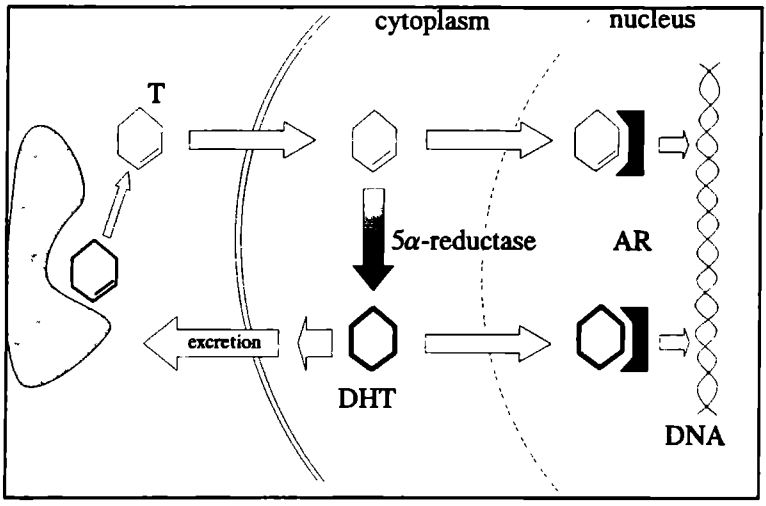

Figure 1.1: Graphical representation of the role of 5α-reductase in androgen action. The non-protein bound fraction of testosterone (T) can readily diffuse across the plasma membrane of a cell. Depending on the machinery in that cell, T can either bind to the nuclear androgen receptor (AR), be metabolized by 5α-reductase to dihydrotestosterone (DHT), which in turn can bind to the same AR, or it can be further metabolized by other enzymes (3-hydroxysteroid oxidoreductases, sulfatase, etc.) and be excreted.

enzyme [2, 3, 4, 118, 156] and the impressive potential of molecular biological techniques, several puzzling aspects of the ontogeny, expression, function, physiology and pharmacology of 5α-reductase has been elucidated [119, 174].

1.3 ROLE AND FUNCTION OF PUTATIVE MULTIPLE 5α-REDUCTASE ISOZYMES

It was generally assumed that metabolism of testosterone served to inactivate and to promote the excretion of this androgen hormone. One of the enzymes responsible for the metabolism of testosterone, 5α-reductase, was initially characterized in rat liver slices based on the metabolism of desoxycorticosterone [181, 182]. This organ is equipped with a variety of enzymes, mainly engaged in the catabolism of both endogenous substances and xenobiotics into highly polar metabolites which are more easily excreted. As the 3-oxo group of steroid substrates is rendered more susceptible to reduction by 3α- and 3ß-hydroxysteroid oxidoreductases (HSOR) and to sulfation and glucuronylation after 5α-reduction [14], 5α-reductase was thought to participate in the *catabolism* of steroids.

However, as the latter enzyme would not catalyze the back reaction (dehydrogenation) – although this was originally contended [57] – it was hypothesized that 5α-reduction was a regulatory step [227]. It had already been established that DHT was a more potent androgen than testosterone in bioassays [177]. After administration of radiolabelled testosterone to rats, tritiated DHT accumulated in the nuclei of the prostate [1, 22]. Furthermore, DHT was shown to bind preferentially to specific nuclear (androgen) receptor proteins [41, 127]. Altogether, DHT appeared a more potent androgen than testosterone and accumulated in the presumed subcellular compartment of androgenic action, the nucleus of androgen target cells. Thus, the enzyme responsible for the formation of DHT, 5α-reductase, could play an important role in the augmentation of the *anabolic* effects of testosterone

Originally, it was surmised that at least five distinct isoforms of 5α-reductase were expressed in rat liver tissue, based on the differential regulation of the reduction of specific substrates [136]. Two different isoforms with specific pH-optima and kinetic properties were found in cultured human fibroblasts of which one was absent in pseudohermaphrodites [139, 143]. As both testosterone and cortisol reduction were impaired in patients with male pseudohermaphroditism it was proposed that one enzyme, responsible for the 5α-reduction of all Δ^4-3-oxosteroids, was mutated in these patients [46]. Analysis of human prostatic tissue 5α-reductase activities also led authors to suggest two isoforms, one in stroma, the other in epithelial cells [167]. However, as it seems that in some patients with male pseudohermaphroditism all 5α-reductase enzymatic activity was impaired, it was subsequently surmised that only one enzyme was responsible [85]. Based on a differential age-dependency and sensitivity to enzyme inhibition of the 5α-reduction of testosterone versus androstenedione in rat prostate, the possible existence of two enzyme forms was again proposed [134]. Only with the isolation of the cDNA's of two 5α-reductase isozymes [2, 3, 4, 156], it was finally established that indeed (at least) two isoforms exist in both rat and human, each with surprising specific characteristics [174]. The affinity constants and tissue-specific expression of the isozymes led authors to propose a *catabolic* role for the type I and an *anabolic* role for the type II isozyme [156].

As mentioned earlier, DHT has been implicated in benign prostatic hyperplasia (BPH) [191, 228]. As patients with male pseudohermaphroditism have been diagnosed with 5α-reductase type II deficiency [2, 93] and these patients have atrophic prostates [86], a specific type II inhibitor, finasteride (Proscar®), was developed for the management

of prostatisms [60]. This inhibitor might also be beneficial in the treatment of prostatic cancer [59]. The proposed anabolic role for the type II isozyme [156] is in line with its reported involvement in the growth of the prostate and in the pathogenesis of BPH.

Involvement of 5α-reductase in skin disorders as male pattern baldness [13], hirsutism [95, 106, 186], and acne [175] might be attributed to the type I isozyme that is expressed in non-genital skin [165, 205]. This is in contradiction with the proposed catabolic role of this isozyme [156] and would implicate the type I isozyme also to participate in the anabolic amplification of androgen action.

1.4 GENE STRUCTURE

The first cDNA for 5α-reductase was isolated from female rat liver by injecting liver mRNA in *Xenopus laevis* oocytes [42], which were subsequently assayed for 5α-reductase enzymatic activity by measuring the metabolism of radiolabelled testosterone [3].

The rat cDNA was then used as a hybridization probe to screen a human prostate cDNA library [4]. A 2.1 kilobase cDNA sequence was identified, from which the protein sequence was deduced. This protein shared 60 % identity in amino acid composition with the rat enzyme. Expression of this enzyme in COS cells gave some unexpected results. The expressed 5α-reductase enzyme exhibited a neutral pH-optimum [4], whereas the tissue from which it was isolated, the human prostate, expresses a 5α-reductase enzyme activity with an optimum at pH 5.0-5.5 [120]. Furthermore, the sensitivity of the human 5α-reductase activity in the prostate for the enzyme inhibitor finasteride was not exhibited by the cloned enzyme, and the gene encoding this enzyme was normal in subjects with 5α-reductase deficiency [93]. This led to the hypothesis that the enzyme expressed by the isolated 5α-reductase cDNA and the prostatic 5α-reductase enzyme activity were in fact two different isozymes [93].

Therefore, the total cDNA of the human prostate was monitored for 5α-reductase enzymatic activity in cultured human cells to screen for another isozyme. After division of the cDNA pool into progressively smaller groups, a single 5α-reductase clone was isolated that was different from the first human 5α-reductase cDNA [2]. Expression of this 5α-reductase clone revealed the acidic pH-optimum and the

sensitivity to finasteride exhibited by the 5α-reductase enzymatic activity found in the human prostate. The gene encoding this type of 5α-reductase was shown to be mutated in subjects with 5α-reductase deficiency [2].

Subsequently, oligonucleotides derived from this second human 5α-reductase cDNA were used in polymerase chain reactions to isolate the rat homologue from mRNA of regenerating prostate [156]. Both the rat and human isozymes were designated type I and type II in the chronological order of their isolation [2, 156].

Similar 5α-reductase isozymes were later also detected in the mouse [174]. Recently, the type I and type II 5α-reductase genes were described in the Cynomolgus monkey with approximately 95 % nucleotide sequence identity with the human genes and coding for isozymes with biochemical characteristics (relative substrate affinities, pH-dependencies and inhibitor sensitivities) closely paralleling those of its human homologues [118]. Mouse, rat and human 5α-reductase isozyme genes all contain five exons separated by four introns [94, 107, 174, 203] (*figure 1.2*). As this arrangement is conserved between species, the isozymes probably originated from an ancient duplication and subsequently diverged into the present isoforms. The human type I gene (SRD5A1) is located on band p15 of chromosome 5, whereas the type II gene (SRD5A2) resides on band p23 of chromosome 2. On the long arm of the X-chromosome (q24-qter) a pseudogene (SRD5AP1) is found which is not believed to encode for a functional 5α-reductase [94].

Information concerning the functional domains of the 5α-reductase enzymes came from the analysis of mutations in the disorder of male pseudohermaphroditism [173, 225]. These mutations have only been found in the type II isozyme [93]. So far, no type I 5α-reductase deficiency has been described. Point mutations in exon 1 of the type II gene (*figure 1.2*) that caused a substitution of arginine for glycine (mutation G34R) led to an enzyme with a dramatically decreased affinity for steroid substrate, suggesting that the amino terminus of 5α-reductase is involved in substrate binding [173]. As another mutation that also led to decreased affinity for substrate (H231R) was later mapped to exon 4, it seemed that both ends of the enzyme are involved in substrate binding [174]. Only a non-linear arrangement of the isozyme could implicate both ends of the protein in steroid binding (*figure 1.2*). Two mutations that disrupt the cofactor binding (G196S and R246W) were found in the carboxyl terminus (*figure 1.2*), suggesting that this part of the enzyme is responsible for the binding of NADPH

[173]. However, this kind of mutations has now also been mapped to exons 2, 3, 4 and 5 [174]. Based on the analysis of these mutations, the cofactor binding domain(s) thus map(s) almost throughout the entire type II isozyme.

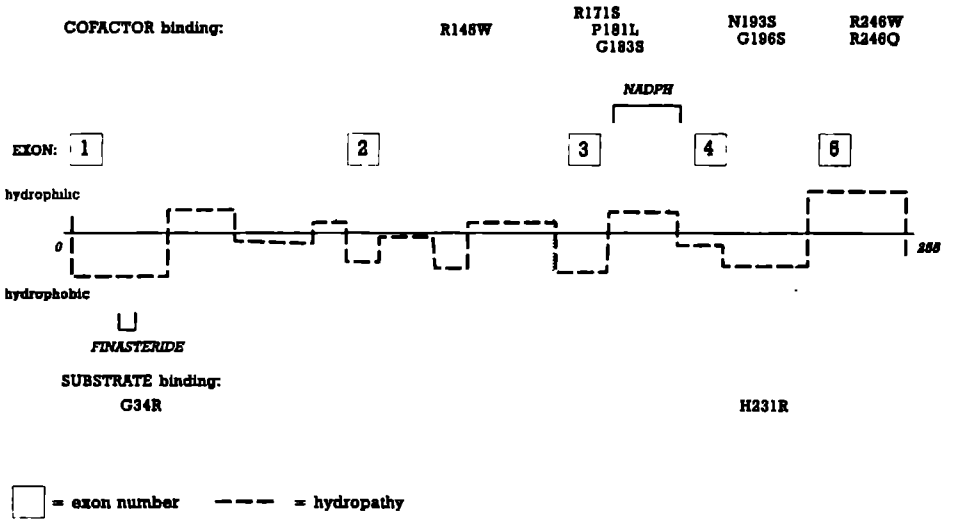

Figure 1.2: Simplified graphic presentation of **i)** the biochemical effects of point mutations in the steroid 5α-reductase gene (denoted by their schematical numbers, for instance R145W: indicating amino acid changed, residue number, and amino acid changed to), either leading to decreased cofactor affinity (above line) or decreased substrate affinity (under line) [173, 225]. **ii)** exon numbering, boxed numbers [3]. **iii)** hydropathy plot [dashed line] (hydrophilic above, hydrophobic under solid line) [3]. **iv)** the cofactor (NADPH) binding domain that binds the probe 2-azido-NADP$^+$ [11]. **v)** the tetrapeptide segment that confers sensitivity to finasteride, and is therefore probably part of the substrate binding domain [204].

1.5 PROTEIN STRUCTURE

From the nucleotide sequences (*section 1.4*) the primary structure of the rat, monkey and human isozymes can be determined. Rat and human share 60 % amino acid sequence identity for the type I and 77 % for the type II isozymes [173]. The monkey type I and type II share approximately 93 and 95 % sequence identity with its human homologues [118].

The isozymes of all three species are highly hydrophobic [3, 118, 173]. The human isoforms are composed of 254 and 260 amino acids with predicted molecular weights of 28 and 29 kDa. No distinct transmembrane regions are found in hydropathy plots, but hydrophobic amino acids are found throughout the protein [3] (*figure 1.2*). This suggests that the isozymes are strongly membrane-bound, and are in fact integral membrane proteins embedded in the lipid bilayer. This explains many problems in the attempts of solubilization and purification of the enzyme [38, 49, 77, 119, 121, 140, 141, 176]. The loss of enzyme activity after solubilization from the membrane suggests an important role for the surrounding membrane in the regulation of 5α-reductase enzymatic activity. Indeed, phospholipases inhibited 5α-reductase activity in rat epididymis [28] and prostate [38] (see *section 1.6.5.2*). The hydrophobic nature of the enzyme may also cause the deviant molecular weights on SDS polyacrylamide gels ranging from 21 to 27 kDa [3, 202, 205].

Remarkably, the rat hepatic 5α-reductase –now considered the type I isozyme [156]– can be solubilized with non-ionic detergents, by high salt treatment, sonication or by repeated freeze-thaw cycles, suggesting it is a peripheral membrane protein, in contrast to the prostatic enzyme [38]. The hepatic enzyme could be solubilized in an active state, whereas prostatic 5α-reductase isozyme activity –type I and type II [156]– was completely inactivated after solubilization [38]. The reason for this tissue-specific difference for rat steroid 5α-reductase is unclear, but suggests a post-transcriptional event for the hepatic isozyme, making it more hydrophilic than its predicted amino acid sequence would indicate. Reportedly, no consensus sequences were identified for either N-linked or for O-linked glycosylation [174], but the human type II isozyme, purified from the prostate, has now been described as O-glycosylated with oligosaccharide side chains containing mannose, N-acetyl galactosamine, fucose, galactose and sialic acids [163]. Such a glycosylation would make the protein more hydrophilic, but this glycosylation has not yet been reported for the hepatic isozymes, nor specifically for the type I isozyme, either from human or rat.

By genetically engineering rat and human type I 5α-reductase chimeric proteins, and exchanging progressively smaller segments, a tetrapeptide-coding sequence in exon 1 of the rat isozyme (residues 22-25) was identified that conferred sensitivity for the 5α-reductase inhibitor finasteride from the rat type I to its human homologue [204]. As finasteride competes with substrate for binding to 5α-reductase, this tetrapeptide (residues 26-29 for the human type I isozyme) is considered to form part of the

substrate binding domain (*figure 1.2*). Surprisingly, this tetrapeptide segment is not completely conserved in the Cynomolgus monkey isozymes, despite the fact that the monkey homologues exhibit biochemical characteristics highly comparable to the human isoforms [118].

No consensus adenine dinucleotide-binding sequences were identified in rat or human 5α-reductase isozymes [174], although the enzyme is totally dependent on NADPH as cofactor. Mutations that alter cofactor binding affinity have been mapped throughout the type II isozyme [174] (*figure 1.2*). The associated amino acids are highly conserved in both isozymes and in the rat homologues, suggesting the cofactor binding domain to be similar in both species. By using polyclonal antibodies against the carboxyl terminus of the human isozymes in Chinese Hamster Ovary (CHO) cells transfected with 5α-reductase cDNA, it was established that the carboxyl terminus of both human isozymes is on the cytoplasmatic side of the endoplasmatic reticulum in these cells [202]. This carboxyl terminus contains a hydrophilic domain (amino acid 232-253 of the rat type I) [3]. Enzyme activity in the transfected CHO cells requires the addition of NADPH when only the cell membrane is permeabilized and the endoplasmatic reticulum is left intact [202], suggesting that the hydrophilic carboxyl terminus of the protein is involved in cofactor binding.

Recently, by labelling of the rat hepatic microsomal 5α-reductase (type I) with [2'-^{32}P]-2-azido-NADP$^+$ [12] and subsequent purification, an 11-amino acid peptide was identified that would be (part of) the cofactor binding domain [11] (*figure 1.2*). Sequence analysis of the purified peptide indicated that it corresponded to residues 170-180 of the rat 5α-reductase type I, that was not equivalent to a consensus adenine dinucleotide-binding sequence [5, 184]. The rat 5α-reductase type I residues 160-190 share approximately 97 % homology with the human type I isozyme (residues 164-194) [11] and the corresponding residues of the monkey type I [118]. The corresponding rat, monkey and human type II isozyme residues show approximately 70% homology vs. the rat type I.

These residues are located in a hydrophilic portion of the rat type I peptide in the exon 3 region according to hydropathy plots [3], and can thus interact with the hydrophilic cofactor NADPH [11]. Several of the mutations described (R171S, P181L and G183S [225] (*figure 1.2*), resulting in decreased affinity for NADPH, lie within the human homologue of this proposed adenine dinucleotide-binding domain. However,

other mutations do not lie within this sequence, emphasizing the necessity of regarding the 5α-reductase isozyme proteins as three-dimensional structures in order to better understand their functional domains.

1.6 ENZYMATIC ACTIVITY

The comparability of 5α-reductase enzyme activity measurements was (and is) hampered by differences in experimental protocol, species and tissues analysed and —originally— the unawareness of the existence of multiple isozymes. This led to a wide range of results in reported enzyme characteristics. In this section several of the characteristics of 5α-reductase *activity* measurements and their intricacies will be discussed.

1.6.1 *pH-optima*

One of the most striking characteristics of the mouse, rat, monkey and human 5α-reductase isozymes is the acidic pH-optimum reported for the type II subtype [2, 93, 118, 143, 156, 174]. In 1970 5α-reductase enzyme activity was reported in homogenates of human foreskin with an optimum at pH 5.5 [217]. Based on the presence of two distinct pH-optima in 5α-reductase activity in human fibroblasts, Moore and Wilson were the first to –correctly– postulate the existence of two isozymes of 5α-reductase [143].

These specific pH-optima can be applied to assign 5α-reductase enzymatic activity in a tissue to a particular isozyme [174]. The acidic pH-optimum for 5α-reductase activity found in genital skin fibroblasts [139, 143] and the more alkaline pH-optimum found in non-genital skin [143] can be attributed to type II and type I isozyme activity respectively, as has been confirmed by Northern blotting and immunoblotting [205]. The ratio of 5α-reductase velocities at pH 7.0 and pH 5.0 have been used as an indication of the ratio of type I and type II 5α-reductase isozyme activities in human tissues [205].

The cloned and expressed human [93] and rat [156] cDNA of type I isozymes specify enzymes which have a broad pH-optimum from 6.0-8.0, whereas the cloned and expressed type II isozymes have a narrow optimum around pH 5.0 [2, 156].
However, reports on the pH-optimum of 5α-reductase activity in *tissues* have yielded

a wide range of results. For the rat prostate pH-profiles have been described with optima at pH 5.0 and around 7.0 [156], pH 6.2 (microsomal) and 6.8 (nuclear) [49], pH 6.5 [120], 6.9 [172], and at 7.2 [189]. For the rat epididymal 5α-reductase, optima have been found at pH 5.0 [156] and 6.2 [137]. Finally, in the human prostate the optimum of 5α-reductase enzymatic activity was found at pH 5.0 [93, 120], 5.5 [132], 6.5 (epithelium) and 7.4 (stroma) [83], and at pH 7.0 [80, 84]. These results were obtained with a variety of experimental protocols, and —in the human prostate— with both normal and diseased tissue. The problem of enzyme inactivation [83] or cofactor depletion [132] at acidic or alkaline pH is not always adequately acknowledged. This could explain at least part of the differences in pH-optima found in literature.

The biochemical basis for the acidic pH-optimum of the rat and human type II isozyme is unclear. However, mutations that alter the affinity for cofactor or testosterone —as in the disorder of male pseudohermaphroditism— can shift the pH-optimum to a more alkaline pH [203], indicating that these mutated amino acids could be implicated in the particular acidic pH-optimum of the type II 5α-reductase. The pH-optimum could reflect a localization of this subtype in an acidic subcellular compartment. However, incubation of CHO cells transfected with type II 5α-reductase cDNA with compounds known to disrupt the pH of internal organelles (NH_4Cl, chloroquine or monensin) did not affect enzyme activities [202]. Thus, the acidic pH-optimum is unlikely to be the consequence of an acidic subcellular localization of the isozyme. Furthermore, gentle permeabilization of these cells with digitonin preserved a pH 7.0 5α-reductase enzymatic activity in transfected CHO cells, which suggests that the acidic pH-optimum might be a result of cell lysis. Finally, a pH-profile of the efficiency ratio, Vmax/Km, indicated that the type II isozyme was most efficient in metabolizing low testosterone concentrations at pH 7.0 [202]. For the microsomal fraction of the human prostate, the Vmax/Km ratio had an optimum at pH 6.0 [40]. The higher efficiency ratio at neutral pH is mainly a consequence of the lower affinity constant, Km, (and not of a higher Vmax) at this pH [40, 202].

1.6.2 *Affinity constants*

1.6.2.1 steroidal substrates

Besides distinct pH-optima, the isozymes of 5α-reductase have dissimilar affinities for steroid substrates like testosterone. Cells transfected with the human or rat type I isozyme exhibit 5α-reductase enzymatic activity with an apparent affinity constant

(Km) for testosterone in cell lysates at pH 7.0 of 1.5 to 5 μM and 1 to 1.5 μM respectively [156, 202, 204]. Cells transfected with the human or rat type II isozyme exhibit enzyme activity, at pH 5.0, with a Km of 230 nM and 74 nM respectively [156, 202]. This 6-20 fold higher affinity of the type II enzyme for testosterone implies that it is better suited to metabolize lower concentrations of testosterone. This difference in Km could also be established for several other steroids like progesterone, androstenedione and, in the rat, corticosterone [156, 202]. Both rat isozymes had a low affinity for cortisol (10-20 μM) [156]. The Km of the rat type I isozyme is consistently at least 2-fold lower than that of the human isoform in transfected cells [204]. However, by exchanging amino acid 22-25 of the rat type I isozyme with those of the human isoform, a similar reduced Km could be found for the chimera relative to the human parent [204]. Thus, this tetrapeptide segment in the amino terminal region of the type I isozyme effects the substrate affinity.

At neutral pH —that is, the optimal pH in plots of Vmax/Km vs. pH [40, 202]— the affinity of the rat, monkey and human type II isozyme for testosterone is in the low nanomolar range (2.5 to 20 nM) [40, 118, 202]. The low endogenous testosterone concentrations [103] or the fairly low concentrations of testosterone produced by the fetal testes are thus best metabolized by the type II isozyme at neutral pH, supporting the hypothesis that this isozyme operates at neutral pH *in vivo* [202].

Differences in affinity constants between 5α-reductase activities in tissues might indicate dissimilar isozyme activities. After mechanical separation of stroma and epithelium of human prostate, 5α-reductase activities with distinct Km's have been reported [21, 83, 103, 167, 222]. These studies reported Km's for testosterone of 23 to 230 nM in stroma and of 11 to 90 nM in epithelial cells. These values are all in the range characteristic for the cloned and expressed type II isozyme, but have led authors to suggest the existence of two different isozymes in the human prostate [e.g. 167]. Measurements in human BPH tissue homogenates yielded a wide range of affinity constants for testosterone varying from 11 nM [21] to 15 μM [120]. As these values are apparent affinity constants because they are obtained *in vitro* and several compounds and experimental procedures are known to influence 5α-reductase enzymatic activity (see *section 1.6.5*), these differences can be attributed to a variety of causes.
In fact, some reports have indicated that only the type II isozyme is expressed in human prostatic tissue [194, 205], although controversies exist [93]. For example,

mRNA for the type I isozyme has also been detected in this tissue [15, 126] and an earlier report on 5α-reductase activities in the human prostate suggests enzyme activity with characteristics (pH-optima, affinity constants) of the type I isozyme [80].

1.6.2.2 cofactor

The affinity constants for the cofactor NADPH has been reported as 3-5 μM for the human type I and 7-10 μM for the type II isozyme in transfected CHO cells [202]. In human liver –probably type I 5α-reductase– the Km for NADPH was 6 μM, in the prostate –probably mainly type II– the Km was 2.5 μM [79]. Therefore, the affinity for the cofactor is approximately similar in transfected cells and tissues. As these values also do not differ significantly between the human isoforms, it is not possible to establish the Km for NADPH so as to distinguish between subtypes in a tissue. However, in rat epididymis the nuclear-bound 5α-reductase activity exhibited an apparent affinity constant of 1 μM for NADPH, whereas the microsomally-located enzyme activity had a Km for NADPH of 30 μM. Both had a Km of approximately 300 nM for testosterone [179]. The pH optima and competitive inhibition profiles were similar for both subcellular fractions, indicating that these two forms are different from those established so far in rat and human. Similarly, in the human prostate 5α-reductase isozymes with a Km of 20 nM for testosterone, but 0.42 and 82 μM for NADPH have been described [110]. Together, this data suggests the existence of a third isozyme with a different affinity for NADPH of 30 (rat) to 82 (human) μM, which, however, has found no confirmation on the protein or mRNA level.

1.6.3 *Mechanism of 5α-reduction*

An earlier report on the mechanism of 5α-reduction in rat liver demonstrated that the electrons are transferred from the cofactor NADPH to coenzyme Q10 via the enzyme NADPH:coenzyme Q10-oxidoreductase, and then to testosterone [57]. If coenzyme Q10 was used as electron-acceptor the reaction was reversible. Subsequent reports, however, failed to inhibit this electron transfer in the rat epididymis and seminal vesicle [27] and in rat prostate [27, 38]. It is now generally accepted that the 5α-reductase enzyme does not require intermediates of electron transfer, but directly transfers electrons from NADPH to T.

The prostatic and hepatic 5α-reductases are considered to follow a sequential kinetic mechanism, which precludes the utilization of an electron transport system. The

proposed mechanism [16, 79, 117] of the 5α-reductase reaction states that the cofactor NADPH is the first to bind to 5α-reductase and that $NADP^+$ is the last to leave the enzyme. It thus includes the steps as shown in figure 1.3.

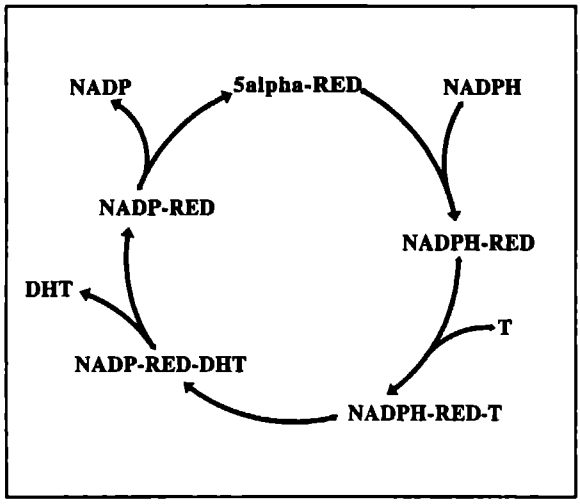

Figure 1.3: Sequential kinetic mechanism of the 5α-reductase reaction. The cofactor NADPH is the first to bind and $NADP^+$ is the last to leave the enzyme. RED is the enzyme 5α-reductase, T is the substrate testosterone and DHT is the product dihydrotestosterone [16, 79, 117].

1.6.4 *Allosterism, cooperativity, hysteresis*

The high degree of specificity exhibited by enzymes prompted Fisher in 1894 to suggest a *lock-and-key* analogy of enzyme-substrate interaction [cited in 185]. The enzyme possesses a region, the substrate binding domain, that is complementary to the substrate molecule. After binding of the substrate, the enzyme can convert it to a product. It is now recognized that this is a simplification [185]. According to the lock-and-key analogy testosterone should be able to bind to 5α-reductase before the cofactor, NADPH. As this is not the case (*figure 1.3*), this analogy does not apply to 5α-reductase. Another hypothesis for the enzyme-substrate interaction is the *flexible enzyme* or *induced fit* hypothesis [101]. The cofactor will induce a conformational change in the enzyme that results in a precise alignment of the catalytical groups of the enzyme with the susceptible bonds of this substrate. After binding of the cofactor

the conformational change in the 5α-reductase enzyme makes it susceptible to testosterone binding. The concept of a flexible enzyme is the basis for theories on allosterism [185], which indeed seems to apply to 5α-reductase [111].

An in-depth analysis of the 5α-reductase kinetics in the particulate fraction of human prostatic tissue revealed that the enzyme exhibited non-michaelian behavior at acidic pH [111, 132], showing an 'initial burst' in the time course of testosterone metabolism [111], which could be attenuated with ATP or dephosphorylating agents [112]. Such an 'initial burst' is described by the term 'hysteresis' [50, 51] and is a consequence of a slow transient in enzyme activity, changing from a higher to a lower steady state rate of activity on a measurable time scale. This hysteretic behavior can be the consequence of a ligand-induced slow transition of the enzyme into another activity state, and can exist with and without cooperativity [155]. The physiological function of such a non-michaelian behavior would be the buffering (damping) of DHT production against oscillations in T concentration [111, 155].

5α-Reductase also exhibited negative cooperativity in acetate buffer at pH 5.5, but not in citrate buffer [111]. The lack of negative cooperativity in citrate buffer in contrast to other buffers is also exhibited by yeast hexokinase [188]. Negative cooperativity is a consequence of the binding of one substrate molecule influencing the binding of the next, either in oligomeric or monomeric enzymes [154]. This makes the enzyme responsive over a wider range of substrate concentration than it would be for a 'michaelian' response [155].

1.6.5 *Regulation*

1.6.5.1 cations

Early reports indicated possibly regulatory effects of cations on 5α-reductase enzymatic activity in the rat prostate [49], and epididymis [137], and the human prostate [80] and epididymis [33]. In rat prostatic nuclear fraction, at pH 6.6, Hg^{2+}, Cu^{2+} and Zn^{2+} exhibited a potent inhibitory effect on 5α-reductase enzymatic activity [49]. Intraprostatic injection of male rats with 10 or 20 mg of zinc-arginine ('neutralized zinc') *in vivo* led to reduced prostatic weight and 5α-reductase enzymatic activity [39]. In the microsomal fraction of rat epididymis, at pH 6.2, the same effect of these cations was established [137]. In human epididymis, at pH 5.3, 5α-reductase was inhibited by Cu^{2+}, Zn^{2+}, Cd^{2+} and Ba^{2+} [33].

In the human prostate, Zn^{2+} was shown to exhibit a dual effect on enzyme activity, stimulating at lower concentrations (2 μM) and inhibitory at higher concentrations (2 mM) [61, 66, 80, 108, 219], with a stronger stimulatory effect on epithelial enzymatic activity, but a more inhibitory effect on stromal 5α-reductase [83]. Zinc is present in the prostate at a concentration of 0.1 mM [195]. Concentrations of zinc are altered in plasma [226] and prostates [161] of patients with BPH. In adenomas of the human prostate the concentration of zinc is increased [183, 206]. Sinquin and colleagues showed that the stimulation of 5α-reductase activities in human prostatic homogenates by Cd^{2+}, Cu^{2+} and Zn^{2+} resulted from augmentation of Vmax, but not from changes in Km [195]. A physiological role for zinc in the regulation of 5α-reductase enzymatic activity and possibly in the pathogenesis of BPH and/or prostatic cancer has been proposed [70].

Recently, it has been shown that the human type II isozyme, expressed in SW-13 cells, is inhibited only by Cu^{2+} (K_i = 11 μM), whereas the expressed human type I isozyme is inhibited by Cd^{2+}, Cu^{2+} and Zn^{2+} with K_i values of 0.61, 3.47 and 1.51 μM respectively [198].

The addition of EDTA in *in vitro* incubations led to a potent stimulation of 5α-reductase enzymatic activity in homogenates of whole human prostate at pH 7.4 [80] and in stromal (pH 7.4) or epithelial (pH 6.5) fractions of this tissue [83]. However, this stimulatory effect of EDTA was shown to result from an EDTA-induced decrease of pH of the incubation medium, and not from the capacity of EDTA of binding cations [195]. The aforementioned effects of cations and/or EDTA will undoubtedly have contributed to the wide range of results concerning 5α-reductase characteristics in literature.

1.6.5.2 membrane environment

Attempts to solubilize and purify 5α-reductase have always met great difficulties as enzyme activity was rapidly lost after chromatography [38, 49, 77, 119, 121, 140, 141, 176]. The strong membrane-bound nature of 5α-reductase may be responsible for these difficulties in purifying the enzyme, and may indicate a role of the membrane environment in stabilizing or modulating enzyme activity.

Specific phospholipids, phosphatidylcholines containing either unsaturated or saturated acyl chains of 12 carbon atoms, were shown to enhance the activity of

solubilized 5α-reductase from rat epididymis [28]. On the other hand, certain polyunsaturated fatty acids were shown to inhibit 5α-reductase activity from rat and human liver microsomes, and from the human prostatic cancer cell-lines LNCaP and PC-3 [122]. Furthermore, the modulatory effect of phosphatidylcholines on solubilized 5α-reductase enzymatic activity from rat epididymis was later shown to strongly depend on the length of the acyl-chains, the long chains being stimulatory, the short inhibitory [99].

In line with these results, it was shown that phospholipases also modulate 5α-reductase enzymatic activity. Phospholipases C and A_2 increased the Km of rat epididymal 5α-reductase activity [28]. Prostatic 5α-reductase activity was more inhibited by phospholipase C and A_2 treatment than was hepatic enzyme activity [38].

Together, these results suggest a role for the membrane environment in the regulation and modulation of 5α-reductase enzymatic activity. Thus, differences in 5α-reductase characteristics in separate tissues could arise from variations in membrane phospholipid composition in these tissues, and complicate the assessment of isozyme activities.

1.6.5.3 steroids/cofactor

The expression and enzymatic activity of 5α-reductase are, amongst others, regulated by estrogens and/or androgens. Estrogens have been implicated in the promotion of prostatic growth [8, 34, 65, 221]. A direct inhibitory effect of estrogens on 5α-reductase activity in prostatic tissue *in vitro* has been reported, although only at high, non-physiological concentrations [6, 96, 97, 104, 157, 189]. This direct effect of high concentrations of estradiol on 5α-reductase activity has also been established *in vitro* in pubic female skin [24]. At low concentrations, estradiol enhances 5α-reductase activity in cultured prostatic explants, but reduces 5α-reductase activity in the prostate of adult rats [130]. This effect was not found in castrated rats. Treatment with estrogens (diethylstilbestrol, 1 mg per day for three years) led to a strong suppression of type II 5α-reductase in epididymis of man [193]. It has been suggested that the inhibitory effect of estradiol on prostatic 5α-reductase in intact rats was the result of the reduction of the concentration of circulating androgens, direct or indirect by LH-suppression [130]. As estrogens are not substrate for 5α-reduction, a direct competitive inhibition is unlikely. A variety of steroids with a Δ^4-3-oxo-structure can serve as substrate for 5α-reductase [174] and thus act as competitive inhibitors, as has been

described for progesterone [24, 33, 211, 212, 217].

Castration of rats led to a marked regression in size and weight of the ventral prostate [141] by apoptosis of the epithelium [164]. Treatment with the 5α-reductase inhibitor finasteride also caused atrophy and apoptosis of epithelial cells in the prostate of rats, although to a lesser extent than castration [169, 187]. Prostatic 5α-reductase enzymatic activity decreases after castration and can be restored by administration of testosterone, DHT [55, 141, 156] and of 3α-androstanediol [141]. Neither administration of estradiol, nor adrenalectomy or hypophysectomy attenuated the effect of suppletion with testosterone on prostatic 5α-reductase after castration [141]. Finasteride blocks the induction by testosterone of prostatic 5α-reductase enzymatic activity and of type I and type II specific mRNA in castrated rats [3, 55, 156]. Therefore, it can be concluded that DHT per se induces both type I and type II 5α-reductase expression. This positive-feedback regulation mechanism is characteristic for a number of genes that play crucial roles in development [174]. The formation of trace amounts of DHT would induce 5α-reductase expression, increase DHT formation, and thus amplify a positive developmental cascade [55].

Pituitary [207] and adrenal [114] 5α-reductase activities seem to be regulated differently from the prostatic enzyme. Pituitary 5α-reductase is controlled by both direct and indirect gonadotrophin-releasing hormone-mediated mechanisms. Castration led to enhancement of 5α-reductase enzymatic activity, whereas administration of testosterone decreased pituitary 5α-reductase activity in both intact and castrated rats [207]. Castration of male rats led to a threefold increase in adrenal 5α-reductase activity and a sevenfold increase in 5α-reductase mRNA that could be restored to normal values by DHT administration. Pregnant mare serum gonadotrophin decreased adrenal 5α-reductase activity in immature female rats [114]. Thus, pituitary and adrenal 5α-reductase activities are suppressed by androgens, which is in marked contrast with the prostatic enzyme activity.

DHT has been shown to non-competitively inhibit 5α-reductase activity *in vitro* [79]. However, other reports deny product inhibition of 5α-reductase [111]. The reported non-competitive inhibition by $NADP^+$ [16, 79, 117] is in line with the proposed sequential ordered mechanism of 5α-reductase (see *section 1.6.3*). One could speculate on a possible regulatory role for NADPH and/or $NADP^+$ *in vivo*, especially as NADPH is also considered to stabilize enzyme activity [115, 120, 225]. In any

case, the possibility of product inhibition by DHT or $NADP^+$ is a matter of consideration in the assay of 5α-reductase.

1.6.5.4 other regulatory factors

A regulatory factor of 5α-reductase enzymatic activity that could play a role *in vivo* or *in vitro* (in the assay of this enzyme) is citrate [111, 132]. Citrate, a commonly used buffer-component, is present in prostatic epithelium and is in fact a major secretory component of it [30]. Tissular citrate concentrations are elevated in BPH, but low in prostatic carcinoma [30, 31]. Citrate as buffer component was shown to abolish the negative cooperativity exhibited by BPH 5α-reductase activity when assessed in acetate buffer [111] (*section 1.6.4*). Prostatic citrate metabolism is possibly regulated by testosterone and prolactin [31]. The 5α-reductase activity in testes of both immature and mature rats has been reported to be stimulated by prolactin, both *in vivo* and *in vitro* [26, 200]. Furthermore, ATP was shown to stimulate 5α-reductase activity, whereas DNP (2,4-dinitrophenol, a reagent that lowers intracellular ATP levels [74]) inhibited enzyme activity in BPH microsomes [112]. In the same study, a negative correlation was established between PAP (prostatic acid phosphatase) content and 5α-reductase activity. PAP is located in the epithelial cells of the prostate [196], suggesting that 5α-reductase can be regulated by phosphorylation of the enzyme in the human prostate *in vivo*. Variation in established enzyme activities between different BPH biopsies or discrepancies in literature on 5α-reductase activities may be due to differences in citrate, ATP, PAP [111, 112, 132] or zinc (*section 1.6.5.1*) content of the prostate.

Gonadotrophins reportedly stimulate 5α-reductase enzymatic activity in homogenates of whole testis [147], interstitial cells [153], cultured Leydig cells [148] and in cultured Leydig cell precursors [151]. Platelet derived growth factor (PDGF) inhibits gonadotrophin-stimulated 5α-reductase activity in immature Leydig cells [150], whereas insulin-like growth factor-I (IGF-I) and transforming growth factor ß (TGF-ß) were implicated in the DHT-mediated induction of 5α-reductase activity [76, 218].
The negative cooperativity and hysteresis exhibited by 5α-reductase [111, 112] and described in *section 1.6.4* will not only have a physiological function (damping of DHT production against T oscillations and making the enzyme responsive over a wider concentration range [154, 155]), but also has major consequences for the correct measurement of 5α-reductase enzymatic activity [132]. Finally, Kaufman and colleagues found the 5α-reductase enzyme to influence the behavior of testosterone-

androgen receptor complexes, by coupling with this hormone-receptor complex [98]. What the rationale or effect of this mechanism is, is still to be resolved.

1.7 LOCALIZATION

The localization of 5α-reductase isozymes has been the subject of ample research, as this could shed more light on the possibly distinct roles of these isozymes. This section addresses the localization of 5α-reductase isozyme-specific mRNA and immunoreactivity, and of 5α-reductase enzymatic activities. The isozyme identity of enzymatic activity is difficult to ascertain, as both isozymes catalyse the same reaction. So far, tissular 5α-reductase isozyme activities have been usually identified by their specific pH-optima, although these optima are most probably non-physiologic.

1.7.1 *Tissue distribution*

mRNA

5α-Reductase is expressed in a wide variety of tissues (*table 1.1*). Both type I and type II mRNA were found in rat prostate and epididymis [9, 156]. The expression of 5α-reductase mRNA in rat epididymis precedes detectable enzymatic activity in this tissue, suggesting a posttranslational control mechanism [213, 214].
In human, both type I and type II mRNA's have been detected in the prostate [15, 126], although other authors reported only type II 5α-reductase mRNA in this tissue [125, 205].

Immunoreactivity

Western blots of extracts of rat prostate and immunocytochemical studies in ventral prostate and epididymis revealed immunoreactivity that can probably be attributed to type I 5α-reductase [75], whereas both type I and type II specific antisera resulted in staining of regenerating rat ventral prostate sections [9] (*table 1.1*).
The rat type I-specific antiserum stained human prostatic sections [75]. Type II 5α-reductase immunoreactivity was detectable in human prostate [37, 193, 194, 205].

Enzymatic activity

5α-Reductase enzymatic activity has been demonstrated in the prostate and epididymis of lion, dog, mouse, guinea pig, bobcat and bull [56].
In rats, enzyme activity was found in liver [135, 136], neural tissues [7, 10, 25, 210],

prostate, epididymis [156], lung, seminal vesicles, kidney, submaxillary glands, testis, spleen, skin and uterus [56, 210]. The enzymatic activity in the rat prostate exhibits two pH-optima, one at acidic pH (≈type II) and one at neutral pH (≈type I). The rat epididymal 5α-reductase activity is optimal at acidic pH, indicative of type II 5α-reductase [156]. Neuronal 5α-reductase enzymatic activity is, amongst others, implicated in the formation of 5α-dihydroprogesterone, which is a potent natural ligand of the γ-amino-butyric $acid_A$ receptor [72, 100, 129]. However, formation of DHT is also considered important in the mammalian brain and pituitary [133].

5α-Reductase enzymatic activity can be found in numerous tissues of the rhesus monkey (*Macaca mulatta*). In the male monkey the adrenal, testis, prostate, seminal vesicle, intestine, kidney, liver, lung, foreskin, mesenteric fat, pituitary, cerebellum and cerebral cortex exhibited 5α-reductase activity [131]. In the female monkey, this distribution was similar *p.p.*, and further included the ovary, endometrium and myometrium [131]. In fetal neural tissues, heart, muscle and lung, 5α-reductase activity was also detectable [190]. Based on its acidic pH-optimum, the 5α-reductase activity in the cynomolgus monkey (*Macaca fascicularis*) prostate has been identified as type II [118].

In man, 5α-reductase activity can be found in genital (acidic pH-optimum) and non-genital skin (neutral pH-optimum) [13, 58, 143, 230], prostate [20, 43, 56], epididymis [33, 56], ovary, adrenal, kidney, seminal vesicle, testis, hypothalamus, pons, cerebellum and liver [174, 205]. The pH-optimum in the human prostate is acidic, indicative of type II 5α-reductase [20, 43, 56]. The 5α-reductase activity in cultured beard dermal papilla cells also exhibits an acidic pH-optimum, whereas the papilla cells derived from occipital scalp hair exhibit a type I -like (neutral) pH-optimum [90]. As mentioned earlier, the ratio of enzymatic activity at pH 7.0 over that at pH 5.0 was used to estimate type I / type II activity in human tissues [205]. The adrenal, hypothalamus and pons only exhibited activity at pH 7.0 (type I), whereas the testis would express only type II activity. Other tissues express a variety of pH 7.0 over pH 5.0 activity ratios [205].

Table 1.1: Tissue distribution of 5α-reductase isozyme type I and type II mRNA and immunoreactivity.

species	type I		type II	
	mRNA	**immunoreactivity**	**mRNA**	**immunoreactivity**
rat	prostate[9,55,156] epididymis[9,156,213,214] liver[9,156] testis[215], adrenal,[113,156] seminal vesicle, brain, colon, intestine, kidney, ovary, lung, muscle, spleen, stomach	prostate[9,75] epididymis[37,75,216] liver[37,75,178] testis[215] seminal vesicle[75] brain[160]	prostate[9,156] epididymis[9,156] testis, adrenal,[113,156] seminal vesicle, brain, colon, intestine, kidney	prostate[9]
monkey	prostate, epididymis[118,126] liver, skin, testis		prostate,epididymis[118,126] liver	
human	prostate[15,126] liver[126,205] brain, non-genital skin[205]	(prostate)[75]* liver[194,205] non-genital skin[205]	prostate[15,125,126,205] epididymis[205] liver[126,205] seminal vesicle[205]	prostate[37,193,194,205] epididymis[194,205] liver[37,194,205] seminal vesicle, genital[37,205] skin, cerebral cortex, kidney, ovary, oviduct, uterus, placenta, mammary gland, salivary gland

* with rat type I antiserum

1.7.2 *Cell-type specific localization*

mRNA

In regenerating ventral prostate, type I antisense RNA probes demonstrated labelling of the basal epithelial cells, whereas a type II probe led to almost exclusive staining of stromal cells [9] (*table 1.2*). Both type I and type II mRNA's were expressed in the epithelial cells of the rat epididymis [9], in a gradient, i.e. highest in the segment closest to the testis (initial segment) and decreasing towards the tail of the gland (cauda epididymis) [156, 213, 214]. In the rat liver, mRNA for the type I 5α-reductase subtype was also found to be expressed in a spatial gradient, being highest in hepatocytes surrounding the portal triads and lowest around the central veins [9]
In the human prostate both type I and type II mRNA's have been found [15, 126], but their cell-type specific localization has not yet been reported. The DU-145 cell-line, derived from a cerebral metastasis of an epithelial prostate cancer, was shown to express only type I 5α-reductase mRNA [35].

Immunoreactivity

The stromal localization of the type II and epithelial localization of the type I isozyme mRNA in regenerating rat ventral prostate was confirmed by their immunoreactivity localization [9] (*table 1.2*). Immunocytochemical evidence substantiated the spatial gradient of type I expression in the rat epididymis [216].
In the human prostate type II immunoreactivity seems to be restricted to (basal) epithelial cells [37]. However, Silver and colleagues reported type II immunoreactivity to be predominantly localized in stromal cells, whereas the type I 5α-reductase protein was not detectable in the human prostate [193, 194].

Enzymatic activity

In human skin, the apocrine sweat gland exhibited the highest 5α-reductase activity, followed by sebaceous glands and hair follicles. The enzyme activity in the epidermis was negligible [199]. The enzyme activity in the sebaceous glands had a pH-optimum of 7.5, at which pH the apparent affinity constant was high (24 μM) [199], indicative of type I 5α-reductase. As mentioned in *section 1.6.2.1*, differences in affinity constants between 5α-reductase activities in tissues might indicate dissimilar isozyme activities. After mechanical separation of stroma and epithelium of the human prostate, 5α-reductase activities with distinct Km's have been reported in these compartments [21, 83, 103, 167, 222].

Table 1.2: Cell-type specific localization of type I and type II 5α-reductase mRNA, and cell-type specific and subcellular localization of immunoreactivity of 5α-reductase isozymes.

species	tissue	type I			type II		
		mRNA	immunoreactivity	subcellular	mRNA	immunoreactivity	subcellular
rat	prostate	basal epithelial[9]	basal epithelial[9,75]	nuclear[9,75]	stromal[9]	stromal[9]	nuclear[9]
	epididymis	epithelial[9]	epithelial[75,216]	microsomal to nuclear[216] nuclear[75]	epithelial[9]		
	liver	hepatocytes[9]		microsomal[37,75] microsomal & nuclear[178]			
human	prostate	epithelial cell-line[35] *	(stromal & epithelial)[75]*	(nuclear)[75]*		basal epithelial[37] stromal[193,194]	microsomal[37] nuclear[193,194]
	epididymis					basal epithelial[37,194]	microsomal[37]
	liver		hepatocytes[194]			hepatocytes[37,194]	microsomal[37,194]

* with rat type I antiserum

These studies reported affinity constants for testosterone of 23 to 230 nM in stroma and 11 to 90 nM in epithelial cells, all in the range characteristic for the cloned and expressed type II isozyme, but suggesting a preferentially stromal localization of type I 5α-reductase in this tissue. The difference in finasteride-sensitivity of epithelial (K_i = 7 ± 3 nM, IC_{50} = 38 nM) and stromal (K_i = 31 ± 3 nM, IC_{50} = 112 nM) 5α-reductase activity [105, 222] might also indicate a preferentially stromal localization of the type I isozyme in the human prostate, although *both* inhibition constants are in the range of type II. The difference in pH-optima of 5α-reductase activity in the human prostate (pH 6.5 in epithelium and pH 7.4 in stroma) would substantiate this hypothesis [83]. In contrast, the epithelium derived cell-line DU-145 exhibited only type I characteristics (pH-optimum, inhibitor sensitivity) [35].

1.7.3 *Intracellular localization*

Immunoreactivity

In rat prostate 5α-reductase type I staining of nuclear components was observed in Western blots [75] (*table 1.2*). Both type I and type II specific antibodies showed intense perinuclear staining in regenerating rat prostatic sections [9]. Immunohistochemical studies in the rat epididymis have shown that the type I 5α-reductase isozyme was located in the endoplasmatic reticulum of epithelial cells [216]. Along the epididymis, this intracellular localization changes, and in the proximal initial segment an infranuclear staining was found [216]. Another report, however, indicated that 5α-reductase immunoreactivity —probably type I— in rat epididymis was nuclear bound [75]. Type I immunoreactivity in rat liver has been reported as either microsomal [37,75] or both nuclear and microsomal [178].

In the human prostate, the rat type I antiserum stained the nuclei of both stromal and epithelial cells [75]. Human type II specific antiserum localized 5α-reductase immunoreactivity in the nuclei of stromal cells of the human prostate [193, 194]. Another study, however, reported type II immunoreactivity preferentially in the cytoplasm of the epithelial cells [37].

Enzymatic activity

In rat epididymis, 5α-reductase activity is found in both the nuclear and microsomal fractions [137, 170]. This intracellular localization of 5α-reductase activity in rat epididymis is dependent on age and differs between epididymal sections [180]. The nuclear and microsomal subtypes differ in cofactor affinity [179], sensitivity to

phospholipase treatment [29], and in effect of addition of phosphatidylserine [99]. The intracellular localization of 5α-reductase activity in the rat prostate has been the subject of extensive investigation. After differential centrifugation, enzymatic activity was found in the nucleus, with a considerable amount of 5α-reductase in mitochondrial and microsomal fractions, ranging from 24 to 74% [38, 49, 140, 142, 210]. The mitochondrial and microsomal 5α-reductase activities might have arisen from contamination of these fractions with nuclear particles, due to the forces during homogenization. Houston and colleagues have shown that in the human prostate this contamination can range from 25 to as high as 90% [78]. The human prostatic 5α-reductase activity is considered to be exclusively nuclear [78, 80, 83].

1.8 3α-HYDROXYSTEROID OXIDOREDUCTASE

One of the major DHT-degrading enzymes in the prostate is 3α-hydroxysteroid oxidoreductase (HSOR, E.C. 1.1.1.50). This enzyme is capable of catalyzing both the reduction of DHT to 5α-androstane-3α,17ß-diol (Adiol) and the back-oxidation of Adiol to DHT. HSOR activities have been described in the rat prostate [47, 52, 109, 124, 208] and in the rat epididymis [73, 162, 170, 180]. This enzyme is responsible for most of the DHT metabolism in these tissues [47, 116, 124], as in the human prostate [92, 105] .

Because of the extensive back-oxidation potential of Adiol to DHT by HSOR [19], it was thought that Adiol could play a role as prehormone for the accumulation of DHT in the prostate, and —as DHT is considered to be the active androgen in the prostate— possibly in the development of human benign prostatic hyperplasia (BPH) [145]. Adiol was indeed shown to induce BPH in the dog, the only other species besides man that spontaneously develops prostatic hyperplasia [221]. Adiol levels correlated positively with prostate size in dogs [92]. However, this induction of prostatic hyperplasia by Adiol might not only have arisen due to its back-oxidation to DHT, but could also be a direct effect of Adiol itself, as Adiol has recently been shown to induce cAMP formation in dog and human prostatic tissue explants [152].

Diminished degradation of DHT by HSOR will also lead to accumulation of this potent androgen. Therefore, it was hypothesized that the activity ratio of DHT-forming and DHT-degrading enzymes might be altered in BPH and would be a more accurate pathogenic factor for BPH than 5α-reductase enzymatic activity *per se* [17, 20].

Endogenous DHT levels were reportedly elevated and Adiol levels decreased in human hyperplastic vs normal prostate [53, 69, 103]. However, HSOR activities in hyperplastic prostates have been reported higher [43, 92] or lower [17, 89] than, or similar to [146] those in normal prostates. Studies indicated that 5α-reductase, as well as HSOR activities were both higher in stroma than in epithelium of the human hyperplastic prostate [32, 71]. Other studies indicated that only 5α-reductase was higher in BPH stroma vs normal stroma, whereas HSOR did not differ between stroma or epithelium of hyperplastic or normal prostates [103]. Localized regional variation in the 5α-reductase- over HSOR-activity ratio has also been described in the human prostate [67].

HSOR activity has been detected in rat prostatic cytosol [88, 201, 208], but also in nuclear fractions [208]. These rat prostatic HSOR enzymatic activities differed in cofactor-dependency and in pH-optimum. In rat epididymis, the pH-profile of HSOR activity was optimal at pH 4.0-4.8 [162]. The NADPH-dependent HSOR activity in rat pituitary had a pH-optimum ranging from 6 to 9, whereas the NADH-dependent activity was optimal at pH 5.5 [102]. In the dog prostate optimal activity was found at pH 4.5-7.0 [91]. HSOR activities with dissimilar characteristics were also found in cytosol vs microsomes of human prostate [81, 82, 92]. The cytosolic HSOR was totally dependent on NADPH as cofactor, whereas the microsomally-bound enzyme could use both NADPH and NADH. In these reports, both enzymes were less active in the degradation of DHT in hyperplastic prostates when compared to normal prostates [81, 82]. Further, the cytosolic HSOR activity was optimal at pH 7.0 to 8.0, whereas the microsomal enzyme exhibited maximal activity at pH 4.5-6.0 [92]. This variety in results strongly suggests the possible existence of multiple isozymes of HSOR, which in turn could cloud the quantification of enzymatic activities in different tissues. As for 5α-reductase, the aforementioned characteristics must all be taken into account for a correct apprehension of HSOR-mediated DHT forming and degrading dynamics [105].

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1.10 SCOPE OF THIS THESIS

Several techniques have been used to identify and quantify the 5α-reductase isozymes. Although *mRNA* measurements (by in situ hybridization or Northern blot) are highly specific, their levels do not always correlate with tissular enzyme activity. *Protein* detection studies (immunohistochemistry, immunocytochemistry, immunoblotting) have the unique possibility to assess the specific localization (tissue-specific, cell-type specific and intracellular, see *section 1.7*) of the isozymes in tissue sections, but this kind of technique is hampered by the high degree of homology between the two isozymes (*sections 1.4 and 1.5*). It is particularly difficult to obtain the necessary specific antibodies for this purpose. To ensure binding of antibodies in tissue sections, the antibodies should be raised against isozyme-specific hydrophilic portions of the isozymes. Furthermore, correct determination of the level of enzyme expression with this method is, at the best, semi-quantitative.

Lack of specificity is also one of the problems associated with 5α-reductase *enzymatic activity* measurements. As both isozymes obviously perform the same reaction, i.e. conversion of testosterone to DHT, discrimination between isozymes is difficult. However, if this problem can be adequately overcome, 5α-reductase activity measurements offer better means of quantifying and comparing the *in vivo* enzymatic capacities of normal and diseased tissues than do the aforementioned techniques.

The data obtained with the aforementioned techniques are complex to interpret (*figure 1.4*). The expression of the enzyme can differ between *tissues*, which could cause lack of correlation between mRNA levels and enzymatic activity. Furthermore, comparing *isozymes* on the mRNA- protein- and enzymatic activity level is also problematic. The control of each of the steps as depicted in figure 1.4 can differ between tissues and/or between isozymes. For instance, high levels of type II 5α-reductase mRNA could be associated with a relatively higher level of type I activity, if this subtype is more efficiently expressed in a tissue. Even the quantification of protein (immunoblotting) is precarious, as the catalytic efficiency from protein to activity might differ between tissues or between isozymes. Finally, for 5α-reductase isozyme activities, several methods have been used to assess their quantification. Normally, enzyme activities are measured at optimal conditions of substrate and cofactor concentrations, temperature, buffer composition and pH. As the isozymes have different pH-optima, isozyme

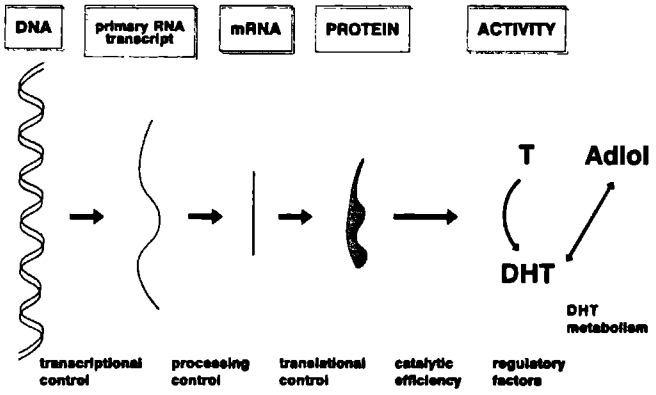

Figure 1.4: Several steps at which 5α-reductase isozyme activities could be differentially controlled. The product of this activity, DHT, is subsequently metabolized by several enzymes, amongst others HSOR.

activities can be quantified at their optimal pH, thus at pH 5.0-5.5 and at pH 7.0-8.0, for type II and type I respectively. However, the supposition that the isozymes operate at neutral pH *in vivo* would argue for measuring isozyme activities at an equal, i.e. neutral pH.

To get more insight into the regulation of tissular DHT concentrations, it has to be taken into account, apart from the intricacies adherent to the quantification of 5α-reductase isozymes, that these tissular DHT concentrations are controlled by a number of degrading enzymes, amongst others 3α-hydroxysteroid oxidoreductase (HSOR). This enzyme can also catalyse the back-oxidation of Adiol to DHT and therefore might play an important role in the control of DHT formation degradation and formation.

The **aim of this thesis** is to assess both 5α-reductase isozyme activities at their physiological pH, i.e. pH 7.0 in three different tissues: **i)** the rat prostate, in which both isozymes have been detected on mRNA, protein and activity level (based on two distinct pH-optima) (*table 1.1*). **ii)** the rat epididymis, in which the type II isozyme has been detected on the mRNA and enzymatic activity level, and the type I isozyme on the mRNA and protein level (*table 1.1*). **iii)** the human prostate, in which the type II isozyme has been found on all three levels, but in which the type I has only been detected on the mRNA level (*table 1.1*).

First, this thesis addresses the intricacies of the kinetics and activity measurements of the type II isozyme in rat prostate and epididymis. The stability of enzymatic activity, the non-linear time course of testosterone metabolism —which precludes correct activity measurements in a single time point assay— and the consequences of the establishment of a pH-optimum of 5α-reductase activity are evaluated (*chapter 2*).

Furthermore, isozyme activities are assessed at neutral pH in rat prostate and rat epididymis. The isozyme activities are obtained by applying a wide range of substrate concentrations and by plotting according to Eadie-Scatchard. This proofed to be an appropriate method to detect and quantify isozyme activities (*chapter 3*).

Type II 5α-reductase is considered to mediate the anabolic effects of testosterone in the human prostate, generating the most potent androgen DHT, and therefore is implicated in benign prostatic hyperplasia (BPH). A specific type II inhibitor, finasteride (Proscar®) has been developed for treatment of patients with BPH. However, as mRNA for type I 5α-reductase has reportedly been found in the human prostate, type I 5α-reductase enzymatic activity might also be present in this tissue, in addition to type II (*table 1.1*). Applying the assay we devised as described in chapters 2 and 3, 5α-reductase isozyme activities are investigated in human hyperplastic prostate tissue (*chapter 4*).

The proposed distinct roles of the isozymes (catabolic versus anabolic) are puzzling in light of the ample expression of type I activity in the classical androgen-target organ, the rat prostate. To get more insight in the roles of the isozymes in androgen-target tissues, the subcellular localization of the isozyme activities is studied (*chapter 5*).

DHT tissular concentrations are primarily regulated by the enzymes 5α-reductase and HSOR. To get a better insight into the dynamics of DHT formation and degradation, HSOR activities —both NADP(H) and NAD(H)-dependent— are quantified in rat prostate and epididymis and combined with the enzyme activity parameters we obtained for 5α-reductase (*chapter 6*).

CHAPTER 2

RAT STEROID 5α-REDUCTASE KINETIC CHARACTERISTICS: EXTREME pH-DEPENDENCY OF THE TYPE II ISOZYME IN PROSTATE AND EPIDIDYMIS HOMOGENATES

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Journal of Steroid Biochemistry and Molecular Biology (1995) 54: 185-192

2.1 SUMMARY

Reevaluating the assay for rat steroid 5α-reductase isozymes in prostate and epididymis homogenates we encountered an extreme pH-dependency of the type II isozyme. The time-course of the metabolism of testosterone (T) to 17ß-hydroxy-5α-androstan-3-one (DHT) at acidic pH shows an *initial burst* when the homogenate is not brought to pH before the start of the incubation. Therefore, the rat type II 5α-reductase isozyme does not follow Michaelian law under these conditions making a single time point measurement invalid. Assessing the pH-optimum of 5α-reduction in both rat prostate and epididymis homogenates we found a strong substrate-dependency: at high substrate concentrations a pH-optimum for the type II isozyme of pH 5.0 was found, whereas at lower concentrations pH 5.5 is optimal. Establishing Vmax (maximum velocities) and Km (affinity constants) for the 5α-reduction of T at pH 4.5 to 8.0, the efficiency optimum Vmax/Km appeared to be pH 5.5 in both prostate and epididymis homogenates. Specifically at acidic pH these kinetic characteristics of the type II isozyme vary manifold. Discrepancies in literature concerning 5α-reductase characteristics can –at least in part– be attributed to the choice of optimal pH, or to pH shifts during the assay.

2.2 INTRODUCTION

Steroid 5α-reductase (5α-reductase E.C. 1.3.99.5) is a NADPH-dependent enzyme capable of 5α-reducing a number of steroids with a 4,5 double bond and a 3-oxo group, including glucocorticoids, progestogens, mineralocorticoids, androgens [1] and non-androgens such as the pheromone precursor 4,16-androstadien-3-one [2, 3].

5α-Reductase can serve an anabolic purpose, catalyzing the conversion of testosterone (T) into the more potent androgen dihydrotestosterone (DHT) [4]. DHT binds with higher affinity to the androgen receptor than does T [5, 6]. DHT has been implicated in the pathogenesis of benign prostatic hyperplasia (BPH), prostate cancer, acne vulgaris, androgenic alopecia and hirsutism [7, 8, 9, 10, 11]. 5α-Reductase can also play a catabolic role, as 5α-reduced metabolites are susceptible to 3α/3ß-reduction. These 3-hydroxylated metabolites can be excreted after subsequent hydroxylation or conjugation.

In 1970 Voigt et al. described a 5α-reductase with a pH-optimum of 5.5 in homogenates of human foreskin [12]. It was not until 1976 that Moore & Wilson postulated the existence of two 5α-reductase subtypes with distinct pH-optima [13], later designated type I and II according to the chronological order in which their cDNA's were isolated [14, 15, 16, 17]. Research on patients with a type II 5α-reductase deficiency (pseudohermaphroditism) has underlined the importance of the type II isozyme in the development of several androgen-dependent organs [18, 19, 20]. This signifies an anabolic role of this type II isozyme. The role of the type I 5α-reductase isozyme is not known as patients with a deficiency have not yet been described, but is assumed to be catabolic [14].

The 5α-reductase isozymes are highly conserved as rat and human tissues both exhibit two subtypes [14, 15, 16, 17, 21, 22]. Amino acid sequence homology between rat and human subtypes is higher than between type I and type II of either species [1]. Both rat and human type II 5α-reductase show a narrow acidic pH-optimum, while both type I isozymes have a broad pH-optimum of 6.0-8.0 [14, 22].

In literature the pH-optimum of surmised type II activity in rat or human tissue homogenates differs from pH 5.0 [14, 22], to 5.5 [12, 20, 23, 24, 25, 26], to 6.2 [27] or even 7.0 [28, 29]. Recent work of Thigpen et al. has shed new light upon the

concept of pH-optimum [30]. They postulated the efficiency optimum Vmax/Km. Unlike the classical pH-optimum which only determines velocities at a single substrate concentration, the efficiency optimum has the advantage of taking into account the substrate-dependency of enzyme reaction velocity. However, apparent affinity constants (Km's) for 5α-reductase isozymes differ widely in literature [cf. 23].

Because of these inconsistencies we set out to reevaluate the assay for both isozymes in two rat androgen target tissues: the epididymis, containing predominantly type II 5α-reductase [1, 14], and the prostate, containing both subtypes [14]. We detected a pH-dependent non-linear time course in the 5α-reduction of testosterone in both rat tissue homogenates which could be resolved by bringing the enzyme to the appropriate pH before the start of the reaction. Furthermore, we demonstrated that the reported controversy about the pH-optimum of type II 5α-reductase could –at least in part– be explained by the substrate-dependency of 5α-reduction. Also, we investigated the pH-dependency of enzyme kinetics of both 5α-reductase isozymes. Our results indicate an extreme pH-dependency of the type II isozyme with regard to enzyme characteristics Vmax, Km and Vmax/Km. This pH-dependency could explain –in conjunction with the intricacy of establishing a pH-optimum– many of the discrepancies in affinity constants and velocities found in literature.

2.3 MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]Testosterone (3.74 TBq/mmol) and [1α,2α(n)-^{3}H]17ß-hydroxy-5α-androstan-3-one (dihydrotestosterone) (2.00 TBq/mmol) were purchased from Amersham (Amersham, U.K.). [9,11-^{3}H]5α-androstane-3α,17ß-diol (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high performance liquid chromatography (HPLC, see below) before use. Testosterone was purchased from Steraloids (Wilton, NH). (L+)-Ascorbic acid, ATP (adenosine 5'-triphosphate disodium salt) and EDTA (ethylenediaminetetra-acetate disodium salt dihydrate) were obtained from Merck (Darmstadt, FRG). Glutathione and α-monothioglycerol (3-mercapto-1,2-propanediol) were obtained from Sigma Chemical (St. Louis, MO). TLCK (Tosyl-lysine chloromethyl ketone) and TPCK (Tosyl-phenylalanine chloromethyl ketone) were from Calbiochem (LaJolla, CA) and Pefabloc was obtained from Boehringer (Mannheim, FRG). Diethylether (p.a.), n-hexane (Lichrosolv) and 2-propanol (Lichrosolv) were purchased from Merck. All other chemicals used were of analytical grade.

Protein levels were determined by a method modified from Lowry [31] against a standard of bovine serum albumin (OHRD 20/21, Hoechst-Behring, Marburg, FRG). The assay was modified for microtiter-plates and had a sensitivity of 25 μg per well. Endogenous steroid levels were determined by radioimmunoassay, as described previously [32]. Steroid concentrations were: testosterone 0.2 nM, androstenedione 0.06 nM and corticosterone 5.79 nM in the prostate homogenate, and testosterone 0.2 nM and androstenedione 0.05 nM in the epididymis homogenate. As these concentrations are low compared to the concentrations employed –and the homogenates are diluted over 20-fold in the final assay– endogenous steroids could not interfere with the measurement of 5α-reductase enzyme activity.

Buffers

Homogenization buffer consisted of 20 mM phosphate (Merck), 1 mM monothioglycerol and 0.25 M sucrose (Merck), pH 7.0. The incubation buffer consisted of 200 mM Tris (2-Amino-2-hydroxymethylpropane-1,3-diol, Merck), citric acid monohydrate (Merck) and 2 mM NADPH tetrasodium salt (Merck) in a final volume of 1 ml, pH 4.5-8.0. Final assay pH was checked in control tubes without tracer, before and after incubation.

Tissue preparation

Wistar rats of 7-13 weeks old (200-250 gram) were killed by decapitation and whole prostates and epididymides were removed, freed of adhering fat and brought in liquid nitrogen for transport. Tissues were kept at -80°C or processed immediately. All subsequent procedures were performed on ice. Tissues were thawed and minced with razor blades into small pieces. Minced tissue was homogenized in ice-cold homogenization buffer with a 7 ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ) with a loose and a tight fitting pestle. The homogenate was filtered twice through nylon netting of 50 and 140 mesh respectively to remove cell debris. By this procedure –without centrifugation– a full homogenate with nuclei and cytosol is obtained. For rat prostate the final preparation contained 29.1 mg protein/ml, while rat epididymis homogenate contained 1.1 mg protein/ml.

5α-Reductase assay

Radiolabelled testosterone in ethanol was brought to final concentration by isotopic dilution with non-labelled steroid in Pyrex culture tubes (borosilicate glass, 12x75 mm, Corning Inc., Corning, NY). Ethanol was evaporated under a mild nitrogen stream at room temperature. Incubation buffer was added and the tubes were put in a shaking water bath at 37°C for at least 10 minutes before the start of the incubation to ensure dissolving of substrate (97%). A tube with cofactor (NADPH) in incubation buffer and a tube with the homogenate were placed in the water bath for 10 minutes to obtain the appropriate temperature. In a number of experiments, performed to optimize the assay, several anti-oxidants or protease inhibitors were added before pre-heating the homogenate, i.e. 10 minutes before starting the incubation.

Immediately after adding the cofactor (200 µl) to the substrate, the incubation was started by adding the pre-heated homogenate (10-50 µl). After 10-30 minutes the incubation was terminated by adding 100 µl of 3 M NaOH. To extract metabolites, 4 ml of ice-cold ether was added, the tubes were capped and shaken. The water phase was frozen in an alcohol bath with dry-ice, the organic phase was decanted and evaporated under nitrogen. Metabolites were dissolved in 100 µl hexane for HPLC.

HPLC

Metabolized steroids were separated on a Hibar LiChrosorb Diol-column (length 250 mm, 5 µm, Merck), equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC-system included a Waters 610 Fluid Unit, a Waters 600E System Controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane-propanol 96:4, v/v) was 1.5 ml/min at a pressure of 680 psi. Radioactivity was monitored with a FloOne Beta Radiomatic A500 radio-chromatography detector (Packard-Canberra Benelux, Tilburg, The Netherlands) with a 500 µl cell and a liquid scintillation flow of 1.5 ml/min (Aqua-Luma, Lumac-LSC, Olen, Belgium). The counting efficiency for tritium was 47%. The percentage formation of DHT and 5α-androstane-3α/ß,17ß-diol were used to estimate 5α-reductase activity. Overall experimental recoveries for testosterone, dihydrotestosterone and 5α-androstane-3α,17ß-diol were 90-93%.

Enzyme characteristics

5α-reductase enzyme characteristics were obtained by the Lineweaver-Burke method with a double reciprocal plot of velocity against substrate concentration at pH 4.5-8.0. Affinity constants (Km's) and maximum velocities (Vmax) were determined and the efficiency ratio Vmax/Km was calculated.

2.4 RESULTS

Enzyme stability

Initially, cofactor was added to the incubation mixture containing substrate, and the reaction was started by adding the pre-heated homogenate. Measured velocities, however, differed with time used to pre-heat the homogenate. The enzyme appeared to be unstable at 37°C. Within 10 minutes only 8 % of the total enzyme activity could yet be recovered. This applied for 5α-reductase isozymes type I and II in both rat tissues. 1 mM monothioglycerol was added in the homogenization buffer beforehand. Neither anti-oxidants (ascorbic acid (50 mg/L) or glutathione (10 mg/L), or both), nor EDTA (1 mM), ATP (2 mM), nor protease-inhibitors (Pefabloc (100 mM), TPCK (200 mM) or TLCK (100 mM), alone or simultaneously), did prevent the

deterioration of enzymatic activity at 37°C. After the enzyme had deteriorated, adding NADPH before starting the incubation did not restore enzyme activity. However, adding NADPH before the 10 minutes pre-heating of the homogenate greatly preserved enzymatic activity. Keeping the homogenate on ice with NADPH until adding it to the incubation mixture gave best results, preserving 80% of enzyme activity. Although the homogenate then would not attain the appropriate temperature at the start of the incubation, we used this protocol in the subsequent study.

Non-linear time course

The time course in metabolism can be considered linear, allowing for a single time point measurement to estimate initial velocities, until substrate depletion leads to a substantial decrease in velocity. Percentual conversion of T to DHT was not allowed to exceed 15%, by varying incubation times with different substrate concentrations, to prevent this substrate depletion. Analysis of the time course of testosterone metabolism, however, showed an "initial burst": in the first 30 seconds the measured velocity was higher than during the subsequent 10 minutes. This phenomenon was observed only at acidic pH in both rat prostate and epididymis homogenates (*figure 2.1A and B*). For the type I isozyme – in rat prostate homogenate at pH 7.0 – the time course of testosterone metabolism was linear (*figure 2.1C*). The initial burst precluded the correct estimation of initial velocities of 5α-reductase type II at acidic pH in a one-time point measurement. In our initial protocol pH was presumably obtained almost immediately, as the homogenization buffer was a 20 mM phosphate buffer, the incubation buffer used was a 200 mM Tris-citrate buffer and only 10 to 50 μl of the homogenate were added to a final volume of 1 ml incubation buffer of the appropriate pH. However, bringing the enzyme to pH before starting the incubation by adding 0.2 ml of incubation buffer of the appropriate pH to the homogenate, the "initial burst" could be prevented (*figure 2.1A and B*).

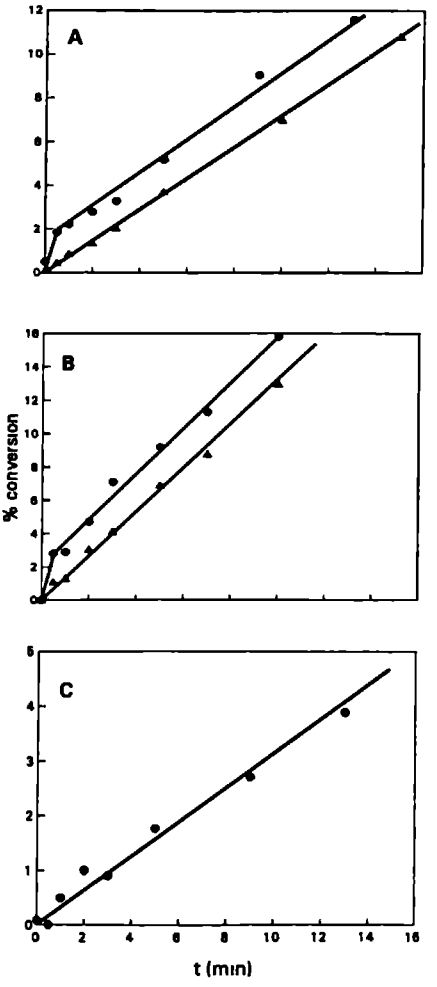

Figure 2.1: Time course in the conversion of T to DHT. A: rat prostate homogenate. B: rat epididymis homogenate. 5α-Reductase was assessed at pH 5.5 using 30 nM T as substrate. When the homogenate is kept in homogenization buffer pH 7.0 an initial burst is found (●). When the enzyme is brought to pH 5.5 by the addition of incubation buffer, the time course is linear (▲). C: In rat prostate the time course at pH 7.0 and using 300 nM T (type I 5α-reductase) is linear (●). The protocol and control of buffer pH are as described in Material and Methods.

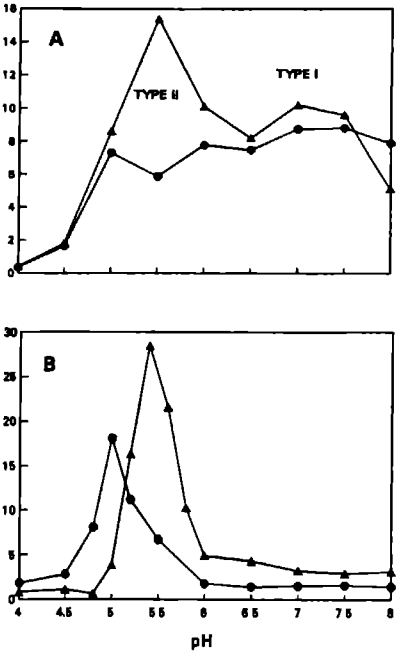

Figure 2.2: pH-dependency of T conversion in: A: rat prostate homogenate. B: rat epididymis homogenate. 5α-Reduction was assessed with 1 μM T (●) or with 10 nM T (▲) at pH 4.0 to 8.0 as described in Materials and Methods. The optimum pH for the type II isozyme at acidic pH is dependent on the substrate concentration used.

pH-dependency of rat 5α-reductase isozymes

pH-profiles showed that rat prostate contains both 5α-reductase isozymes (*figure 2.2A*): enzyme activity can be detected at pH 5.0-5.5, indicative of type II 5α-reductase, and at pH 6.0-8.0, the earlier described optimum of the type I isozyme. Rat epididymis showed high type II activity at acid pH, but only minimal activity at pH 6.0-8.0 (*figure 2.2B*). The type II 5α-reductase isozyme has been reported to have a narrow pH-optimum of either pH 5.0 or pH 5.5. In our hands both pH's were optimal, albeit under different conditions (*figure 2.2A and B*). A careful analysis of substrate dependency at both pH's showed that at testosterone concentrations above 80 nM an optimum of pH 5.0 would be found, whereas smaller amounts of testosterone are metabolized more efficiently at pH 5.5 (*figure 2.3A and B*). The type II isozyme in the rat prostate shows the same substrate-dependency at acidic pH as in the epididymis. In the epididymis only a low maximum velocity –with a high affinity– is attained at pH 7.0 (*figure 2.3B*). In rat prostate homogenate the type I isozyme (at pH 7.0) is more efficient in metabolizing high concentrations of T (*figure 2.3A*).

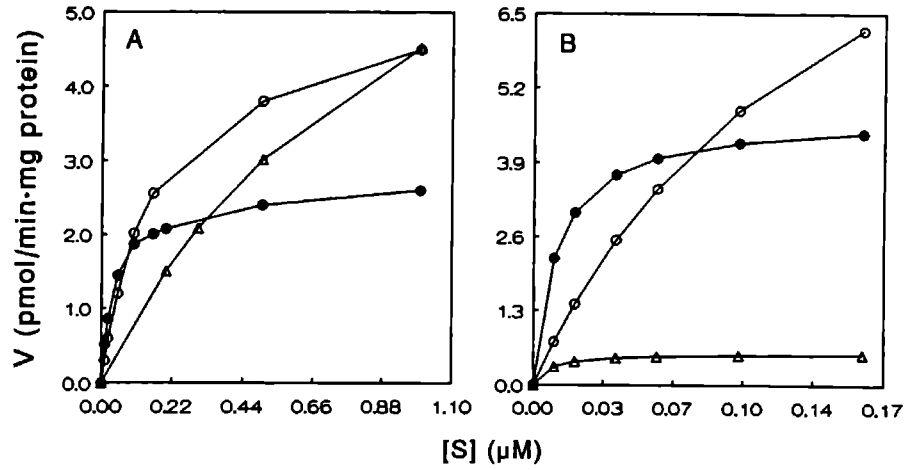

Figure 2.3: Substrate dependency of pH-optimum. A: rat prostate homogenate. B: rat epididymis homogenate. 5α-reductase enzyme activity was assessed at pH 5.0 (-○-), at pH 5.5 (-●-) and at pH 7.0 (-Δ-). At low substrate concentrations in both tissues the highest initial velocity is found at pH 5.5. Higher T concentrations are more efficiently metabolized at pH 5.0. At T concentrations higher than 1μM, pH 7.0 would become optimal in rat prostate, but not in rat epididymis homogenates.

The type I isozyme in rat prostate homogenate has a broad classical optimal pH of 6.0-8.0 (*figure 2.2A*). Enzyme characteristics Vmax, Km and Vmax/Km for this isozyme did not differ substantially in this pH-range (*figure 2.4A, B and C*). The enzyme characteristics for the type II isozyme, however, differed widely. At acidic pH, Vmax and Km showed a strong pH-dependency in both tissues (*figure 2.4*). At pH 4.5 to 6.0 Vmax and Km varied by a factor 20. The efficiency ratio Vmax/Km was also extremely pH-dependent in the acidic range and had an optimum at pH 5.5 in rat prostate and epididymis (*figure 2.4C and F*).

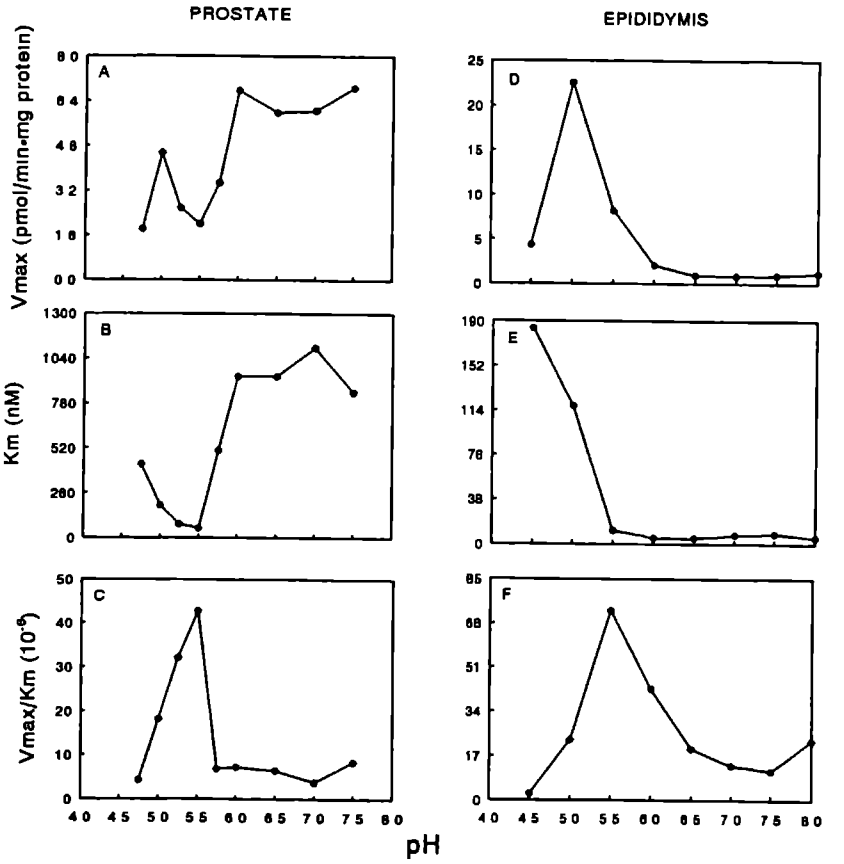

Figure 2.4: Enzyme characteristics Vmax (A, D), Km (B, E) and Vmax/Km (C, F) at pH 4.75-7.5 (rat prostate A, B, C) and pH 4.5-8.0 (rat epididymis D, E, F). Vmax and Km were determined by a double reciprocal plot of estimated initial velocity against substrate concentration according to Lineweaver-Burke. The Vmax vs. pH shows the pH-optimum at high (saturating) substrate concentrations. Vmax/Km plotted vs. pH shows its pH-optimum at pH 5.5 in both tissue homogenates. In both tissues enzyme characteristics vary manyfold, especially at acidic pH.

2.5 DISCUSSION

Several papers have shown the existence of at least two isozymes of 5α-reductase in both rat [14, 16, 21] and human [16, 17, 22] tissues. Most recent research has focused on the measurement of 5α-reductase mRNA and 5α-reductase expressed in transfected cell systems [15, 16, 17, 21, 30, 33, 34]. However, correlation between mRNA level and assayable 5α-reductase activity (protein) is sometimes poor, especially in human tissues [1, 22, 35]. Therefore research at the protein level is still warranted. As kinetic data concerning 5α-reductase differ widely in literature –reported affinity constants of testosterone for 5α-reductase range from 10 nM to 15 μM [23, 30]– we reevaluated the assay of this enzyme in two androgen-dependent rat tissues, the prostate and the epididymis. We used a full homogenate to keep conditions as close to the *in vivo* environment as is possible in this kind of *in vitro* measurements. In rat prostate both isozymes type I (with a physiological pH-optimum) and type II (acidic pH-optimum) are found [14]. In rat epididymis the pH-optimum suggests mainly type II 5α-reductase activity [14], as does the apparent affinity constant at pH 7.0 (see below).

A major problem we initially encountered was the instability of both isozymes at 37°C. Anti-oxidants and protease inhibitors could not prevent this deterioration of enzyme activity and neither ATP nor EDTA did influence 5α-reductase activity. It has been established that NADPH binds to 5α-reductase first, allowing testosterone to bind subsequently [36, 37]. Liang et al. reported that inactivation of human and dog 5α-reductase preparations could be prevented by addition of the cofactor NADPH while homogenizing the tissues [38]. Mutant human 5α-reductase enzymes in both fibroblasts and transfected cells with a shorter half-life than normal enzyme preparations have been reported with a lower affinity for NADPH [1, 4, 18, 20]. A role for the cofactor to stabilize the enzyme in the cell and to regulate enzyme turnover has been suggested [4]. Therefore we investigated the influence of adding NADPH on the encountered enzyme deterioration. In our hands, NADPH pre-binding had a pronounced stabilizing effect on both rat isozymes, but could not restore enzyme activity after pre-heating at 37°C. Several studies have shown the importance of specific membrane components and the effect of phospholipases –which would influence membrane fluidity– on 5α-reductase enzyme activity [39, 40]. During cell homogenization perturbations in the membrane environment of the tightly membrane-bound enzyme might cause a conformational change in the protein. Binding of the cofactor might make the enzyme less susceptible to these membrane perturbations

during homogenization, leaving the enzyme receptive for testosterone binding and metabolism.

Apart from the deterioration of 5α-reductase activity which could be prevented by the addition of NADPH, we encountered a non-linear time course in the metabolism of testosterone at acidic pH in both rat epididymis and prostate (*figure 2.1*). Earlier the group of Martin et al. reported that the 5α-reductase in the particulate fraction of BPH (Benign Prostatic Hyperplasia) homogenates also did not follow first-order Michaelis-Menten kinetics due to a similar non-linear time course at pH 5.5 [23, 41, 42]. This would have a profound effect on the measurement of enzyme characteristics Km and Vmax, when a single time point measurement is used. When we brought the tissue homogenate to the desired pH before starting the incubation, a linear time course was obtained. In rat prostate high substrate concentrations are efficiently metabolized at neutral pH by the type I isozyme. A sudden pH-shift from neutral to acidic pH might explain the initial burst encountered in this tissue, when one assumes that at the start of the incubation a part of the substrate is metabolized by the type I isozyme. cDNA for type I 5α-reductase has been derived from the human prostate, although an acidic pH-optimum of enzyme activity in prostate extracts is found [22], typical for the type II isozyme. Recent evidence however does suggest the expression of type I 5α-reductase activity (P.M. Martin & F.K. Habib, pers. communication). Also in rat epididymis no [1, 14] or only minimal [43] type I 5α-reductase activity is found [see below]. Because of the lack of measurable type I activity in rat epididymis, no higher velocity at any substrate concentration is found at neutral pH in our assay. We believe interference between the two isozymes not to explain the encountered initial burst. Rather this burst is an intrinsic aspect of the type II isozyme per se. The sudden pH-change in the micro-environment of the membrane-bound enzyme, when starting the reaction, might induce a short change in activity by influencing membrane fluidity and/or inducing a conformational change of the isozyme.

In the rat epididymis type I 5α-reductase has been described by immunocytochemistry [43]. Our results, however, are at variance with this finding. At pH 7.0 in the rat epididymis only minimal 5α-reductase activity is found (*figure 2.3B*). Furthermore, the affinity constants we found at pH 6.0-8.0 in this homogenate are very low (approximately 10 nM) (*figure 2.4E*), while the type I isozyme has a characteristic Km for T as high as 1 μM (*figure 2.4B*). For the human type II isozyme, expressed in Chinese hamster ovary cell lines, Thigpen et al. established a high affinity for T of

about 10 nM at neutral pH [30]. Although one has to be aware of the species difference and the difference in experimental conditions (in prostate or epididymis homogenates, and expressed human type II enzyme in CHO cells), we believe that the enzyme activity at neutral pH we found in rat epididymis can be attributed to the type II isozyme. One might argue that the immunocytochemical detectable 5α-reductase type I enzyme is inhibited or is non-active, as also discrepancies between mRNA and enzyme activity have been reported in human BPH-tissue [22]. However, immunocytochemistry is much more sensitive than our biochemical assay and is capable of detecting extremely small amounts of enzyme. Furthermore, type I 5α-reductase is reportedly highly concentrated in the initial segment of the epididymis [43]. We might therefore not have been able to detect this activity in a homogenate of the whole epididymis. Whether this type I activity in the rat epididymis has any physiological significance –considering its low affinity for T– needs to be addressed in studies using discrete segments of this tissue.

The pH-optimum of 5α-reductase activity in rat or human tissue has been the subject of mild controversy. pH-optima in tissues considered to contain mainly type II 5α-reductase differ in literature; an optimum of 5.0 has been described for the type II enzyme in rat epididymis and prostate and in human prostate [14, 22], and an optimum of 5.5 for the human epididymis and BPH tissue, fibroblasts and other human and rat tissues [12, 20, 23, 24, 25, 26]. In addition, an optimum of 6.2 was found in the rat epididymis by Monsalve and Blaquier [27], while the group of Houston et al. reported a pH-optimum of 7.0 in the human prostate [28, 29]. The normal procedure to obtain a pH-optimum is by incubating an enzyme preparation with a single substrate concentration. Velocity, however, depends strongly on substrate concentration.

From the Michaelis-Menten equation: $V = (Vmax*Km) / ([S]+Km)$, where [S] is the T concentration follows: when $S >> Km$, a pH-profile would indicate Vmax vs. pH. And when $S << Km$: $V = Vmax/Km * [S]$. The velocities found are than proportional to Vmax/Km.

In this paper we described the initial velocities at different testosterone concentrations and pH's. At testosterone concentrations below 80 nM a higher initial velocity is found at pH 5.5, whereas at higher testosterone concentrations the optimum in rat epididymis was 5.0. The pH-optimum shift from pH 5.0 to pH 5.5 reported for some

mutant human 5α-reductase enzymes might be explained by the reported change in substrate or cofactor affinity [4, 20]. As both testosterone and NADPH were kept at a constant concentration in these studies, a change in affinity by a mutation in the enzyme can cause the chosen concentration to induce a different pH-optimum, as a different affinity constant implies a different substrate-dependency.

Unlike the classical pH-optimum, the efficiency ratio Vmax/Km takes into account the substrate-dependency of velocity. This ratio reflects also the potential *in vivo* velocity of DHT formation from T, as endogenous T concentrations are much lower than the Km. In our hands, the optimum of the efficiency ratio for the type II isozyme was at pH 5.5 in rat prostate and epididymis. This ratio has been reported to be optimal at pH 6.0 for a microsomal preparation of human prostatic tissue [44] or at pH 7.0 for the expressed human type II isozyme in a hamster ovary cell line [30]. Preliminary results from our laboratory also indicate that the efficiency optimum in human BPH tissue homogenate is at pH 6.5 (unpublished observations). In rat prostate both 5α-reductase isozymes are present, so the Vmax/Km ratio for the rat type II isozyme at neutral pH cannot be determined in this tissue, as at this pH the type I isozyme will interfere. Because of the high Km of the type I isozyme, low efficiency ratios are found at neutral pH in rat prostate. The low efficiency ratio at this pH in rat epididymis, however, can not be explained by the presence of type I 5α-reductase. In contrast to the rat prostate, affinity constants are 100 fold lower at neutral pH in the epididymis. The decreased Vmax/Km ratio at pH 6.5-8.0 in this tissue is caused by the 10 fold lower Vmax at this pH as compared to pH 5.5, with similar affinity constants.

Since we found the enzyme characteristics Vmax and Km to be highly dependent on minor pH-changes in the acidic pH region, the choice of pH has considerable effects on reported kinetic data. Some effects of modifying substances might be explained by even minor shifts in the pH during the assay [as reported in 45 for EDTA].

The present study discloses that in rat prostate and epididymis homogenates the metabolism of T at acidic pH by the type II 5α-reductase isozyme shows a non-linearity in the time course when not brought to the desired pH before the start of the incubation. Therefore estimation of initial velocities at acidic pH in these tissues is incorrect in a single time point measurement. Bringing the enzyme to the appropriate pH before the start of the incubation leads to a linear time course. When a pH-optimum is attained one has to consider the substrate-dependency of enzyme reaction

velocity. At high substrate concentrations a pH-optimum of pH 5.0 is found for the rat type II isozyme, while at low substrate concentrations an optimum of pH 5.5 is found, equalling the Vmax/Km efficiency ratio pH-optimum in rat prostate and epididymis homogenates. The extreme pH-dependency of the rat type II 5α-reductase isozyme strongly influences enzyme characteristics. The choice of pH for the assay of 5α-reductase type II could explain discrepancies in reported affinity constants in literature.

ACKNOWLEDGEMENTS

We thank Dr. J. Weusten for helpful discussions and for the computer program to calculate the enzyme characteristics. We are greatly indebted to Dr. P.M. Martin (Laboratoire de Cancerologie Experimentale, Université Aix Marseille, France) for his critical review of the manuscript.

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CHAPTER 3

KINETIC ANALYSIS OF RAT STEROID 5α-REDUCTASE ACTIVITY IN PROSTATE AND EPIDIDYMIS HOMOGENATES AT NEUTRAL pH: EVIDENCE FOR TYPE I ACTIVITY IN EPIDIDYMIS

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Journal of Steroid Biochemistry and Molecular Biology 57 (1996) in press

3.1 SUMMARY

Immunocytochemical studies and mRNA measurements have shown that the rat epididymis –like the rat prostate– expresses both rat steroid 5*α*-reductase isozymes i.e. type I and II. So far, enzyme activity measurements in rat epididymis homogenates, however, do not support the presence of type I 5*α*-reductase activity. Incubating homogenates of both tissues with a wide range of substrate concentrations, we were able to detect activity of both isozymes in rat prostate and epididymis tissues at neutral pH. In rat prostate the amount of type I activity, as measured by the Vmax at pH 7.0, exceeds that of type II 5*α*-reductase 50-fold. The efficiency ratio, Vmax/Km, of the type I isozyme accounts for 25 % of the total *in vivo* potential activity. A possible anabolic role for the type I isozyme in rat prostate was thus surmised. In rat epididymis the Vmax of type I and type II 5*α*-reductase at pH 7.0 were similar. Comparison of the efficiency ratio Vmax/Km of either isozyme in the rat epididymis, however, suggested that the type II isozyme would play the major role in the 5*α*-reduction of testosterone at physiological concentrations and at neutral pH. The specific localization of the isozymes should be considered to allow for correct quantification of their in vivo contribution to DHT formation.

3.2 INTRODUCTION

Dihydrotestosterone (DHT) is the major androgen formed by 5α-reduction of testosterone (T) in both androgen target- and non-target tissues [1]. The enzyme responsible for this conversion, 5α-reductase (EC 1.3.99.5), is membrane-bound, NADPH-dependent, and capable of 5α-reducing a number of other steroids with a 4,5 double bond and a 3-oxo-group [1].

To date, two subtypes of steroid 5α-reductase with specific pH-optima and inhibitor sensitivities, designated type I and type II, have been described in both human and rat [2, 3, 4, 5, 6, 7]. Their existence is well documented, but has found little rationality. The tissue specific expression and affinity constants of the isozymes has led investigators to propose an anabolic role for the type II isozyme, which is expressed in the prostate and other classical androgen target tissues, and a catabolic role for the type I isozyme, as it is amply detectable in liver tissue [7]. Both the human and rat type I isozymes have a broad pH-optimum of 6.0 to 8.0, whereas the type II isozymes have a pH-optimum of 5.0 to 5.5 [5, 6, 7]. The rationale for the acidic pH-optimum of the type II isozyme has fascinated many researchers over the years. Recently, applying the efficiency ratio Vmax/Km to the question of pH-optimum for human 5α-reductase isozymes expressed in a Chinese hamster ovary cell line, it has been shown that the type II isozyme is capable of metabolizing physiological T concentrations most efficiently at pH 7.0. The acidic pH-optimum of the type II isozyme would be an artifact [8]. Both 5α-reductase subtypes are now thus considered to operate at neutral pH.

We studied the 5α-reduction of T in rat prostate and epididymis homogenates at pH 7.0 to gain insight into the contribution of both isozymes to DHT formation. In rat prostate both subtypes are present and their activities have been reported [7]. In rat epididymis both subtypes have also been described. The type II isozyme is unequivocally present and its activity has been described. Type I 5α-reductase has been detected immunocytochemically [1, 9] and by mRNA measurements [7, 10, 11]. Enzyme activity measurements, however, do not support type I activity in the rat epididymis [7, 9, 11]. Because of this discrepancy between 5α-reductase protein and activity we investigated whether type I activity could be found in rat epididymis homogenates at pH 7.0 using an in-depth kinetic analysis of 5α-reductase activity in this tissue, with the rat prostate as a control.

3.3 MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]T (3.74 TBq/mmol) and [1α,2α(n)-^{3}H]17ß-hydroxy-5α-androstan-3-one (DHT) (2.00 TBq/mmol) were purchased from Amersham (Amersham, U.K.). [9,11-3H]5α-androstane-3α,17ß-diol (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high performance liquid chromatography (HPLC, see below) before use. T was purchased from Steraloids (Wilton, NH). Diethylether (p.a.), n-hexane (LiChrosolv) and 2-propanol (LiChrosolv) were purchased from Merck (Darmstadt, FRG). All other chemicals used were of analytical grade.

Buffers

Homogenization buffer consisted of 20 mM phosphate (Merck), 1 mM monothioglycerol and 0.25 M sucrose (Merck), pH 7.0. The incubation buffer consisted of 200 mM Tris (2-Amino-2-hydroxymethylpropane-1,3-diol, Merck) and citric acid monohydrate (Merck), pH 4.0-8.0 or pH 7.0, and 2 mM NADPH tetrasodium salt (Merck).

Tissue preparation

Wistar rats of 7-13 weeks old (150-250 gram) were killed by decapitation and whole prostates and epididymides were removed, freed of adhering fat and placed into liquid nitrogen for transport. Tissues were kept at -80 °C or processed immediately. All subsequent procedures were performed on ice. Pooled tissues were thawed and minced with razor blades into small pieces. Minced tissue was homogenized in ice-cold homogenization buffer with a 7 ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ) with a loose and a tight fitting pestle. The homogenate was filtered twice through nylon netting of 50 and 140 mesh respectively to remove cell debris. By this procedure –without centrifugation– a full homogenate with nuclei and cytosol is obtained. The pooled rat prostate homogenate was diluted with homogenization buffer to 29.1 mg protein/ml, while rat epididymis homogenate was diluted to 1.1 mg protein/ml.

Protein levels were determined by a modification of the method of Lowry et al. [12] against a standard of bovine serum albumin (OHRD 20/21, Hoechst-Behring, Marburg, FRG). The assay was modified for microtiter-plates and had a sensitivity of 25 μg per well.

5α-reductase assay

Radiolabelled T in ethanol was brought to final concentration by isotopic dilution with non-labelled steroid in Pyrex culture tubes (borosilicate glass, 12x75 mm, Corning Inc., Corning, NY). Ethanol was evaporated under a mild nitrogen stream at room temperature. Incubation buffer (800 μl) was added and the tubes were put into a shaking water bath at 37 °C at least

10 minutes before the start of the incubation to ensure the substrate was dissolved (97 %). A tube with the homogenate (10-50 μl) and the appropriate amount of cofactor (NADPH, 2 mM final concentration) was diluted to 200 μl with 150-190 μl incubation buffer and kept on ice. The incubation was started by adding 200 μl of the homogenate and cofactor mixture to the pre-heated tubes with substrate [13]. After 10-30 minutes the incubation was terminated by adding 100 μl of 3 M NaOH. T metabolism was not allowed to exceed 15 % by varying enzyme amount and incubation time. This 5α-reductase assay protocol was validated and optimized as reported in an earlier paper [13]. To extract metabolites, 4 ml of ice-cold diethylether was added, and the tubes were capped and shaken. The water phase was frozen in an alcohol bath with dry-ice, the organic phase decanted and evaporated under nitrogen. Metabolites were dissolved in 100 μl hexane for HPLC.

HPLC

Metabolized steroids were separated on a Hibar LiChrosorb Diol-column (length 250 mm, 5 μm, Merck), equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC-system included a Waters 610 Fluid Unit, a Waters 600E System Controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane-propanol 96:4, v/v) was 1.5 ml/min at a pressure of 680 psi. Radioactivity was monitored with a FloOne Beta Radiomatic A500 radio-chromatography detector (Packard-Canberra Benelux, Tilburg, The Netherlands) with a 500 μl cell and a liquid scintillation flow of 1.5 ml/min (Aqua-Luma, Lumac-LSC, Olen, Belgium). The counting efficiency for tritium was 47 %. The percentage formation of DHT and 5α-androstane-3α/ß,17ß-diol was used to estimate 5α-reductase activity. Overall experimental recoveries for T, DHT and 5α-androstane-3α/ß,17ß-diol were 90-93 %.

Calculation of enzyme characteristics

Velocities were plotted against T concentration, and Km and Vmax were calculated using a non-linear regression procedure based on the Michaelis-Menten equation on a PC with the software-program Enzfitter. A double reciprocal plot of the obtained estimated initial velocities against substrate concentration was used. This Lineweaver-Burk plot appeared to be non-linear only at high substrate concentrations. An Eadie-Scatchard plot of velocity over substrate concentration against velocity was used for a more even weighting of points [14] and was non-linear over practically the whole substrate concentration range tested. Two 5α-reductase enzyme activities with a different Vmax and Km could be calculated using the same non-linear regression procedure with fitting to least squares, based on a Michaelis-Menten equation for two isozymes. For the use of the Vmax/Km ratio as an index of potential enzyme activity one has to consider that endogenous T concentrations are much lower than the Km of either 5α-reductase subtype [15]. Therefore applying $Km >> [S]$ in the Michaelis-Menten equation gives: $V = Vmax/Km*[S]$. So, at physiological T concentrations, the enzyme reaction velocity is proportional to Vmax/Km.

3.4 RESULTS

In this study we investigated the 5α-reductase characteristics of rat prostate and epididymis homogenates. The validity of the 5α-reductase assay was checked and reported in an earlier paper [13]. With this assay, the time course of the 5α-reduction of T in these rat tissue homogenates was linear and the enzyme stable for at least 30 minutes at 37 °C with 2 mM NADPH. Therefore, the velocities measured can be considered valid estimates of initial velocities.

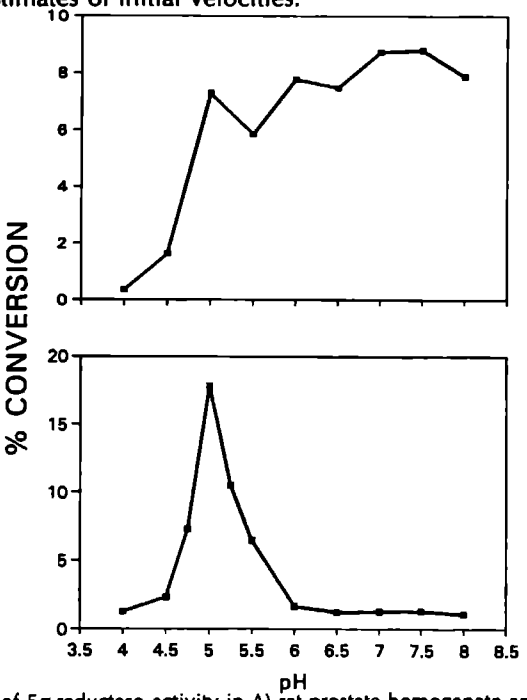

Figure 3.1: pH-profiles of 5α-reductase activity in A) rat prostate homogenate and B) rat epididymis homogenate in the pH range 4.5 to 8.0 with 2 mM NADPH as cofactor and 1 μM T as substrate. The pH-profile in rat prostate suggested the presence of the two established 5α-reductase isozymes, i.e. type II 5α-reductase activity at pH 5.0 and type I 5α-reductase from pH 6.0 to 8.0. In the rat epididymis a pH-optimum was found at pH 5.0, indicative of type II 5α-reductase activity.

In rat *prostate* homogenate 5α-reductase activity was determined with a single substrate concentration of 1 μM T in the pH range of 4.0 to 8.0 (*figure 3.1A*). The two pH-optima suggest the presence of two isozymes in this tissue as the type II 5α-reductase isozyme is primarily responsible for the 5α-reductase activity at pH 5.0, whereas the type I isozyme has a broad pH-optimum from 6.0 to 8.0.

Enzyme activity for 5α-reductase was further established at pH 7.0 with substrate concentrations ranging from as low as 2 nM to as high as 3.2 μM (*figure 3.2A*). A

double reciprocal plot of these data was non-linear at high substrate concentrations (*figure 3.2B*). For a more even weighting of points, an Eadie-Scatchard plot of V/S against V was used (*figure 3.2C*).

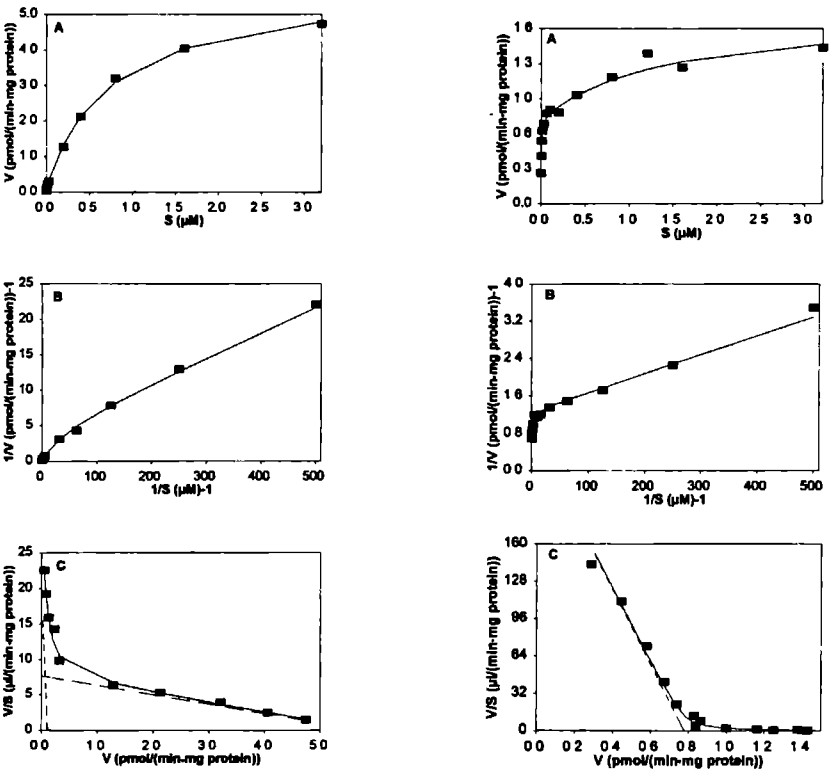

Figure 3.2: A) Estimated initial velocities (V) of 5α-reductase activity in rat prostate homogenates at pH 7.0 with 2 mM NADPH and with 2 nM to 3.2 μM T as substrate (S). Values are mean of two duplicate assays carried out on different days. B) A double reciprocal plot of these data was non-linear at high substrate concentrations. C) An Eadie-Scatchard plot of estimated initial velocities over substrate concentration (V/S) against velocity (V) in rat prostate homogenates at pH 7.0. This plot gives a more even weighting of points and is non-linear. It could be described by two enzyme activities, an alleged type I (—) and type II (...) 5α-reductase isozyme. The abscissa intercepts give the respective Vmax's, while the slope indicates $-Km^{-1}$.

Figure 3.3: A) Estimated initial velocities (V) of 5α-reductase activity in rat epididymis homogenates at pH 7.0 with 2 mM NADPH as cofactor and 2 nM to 3.2 μM T as substrate (S). Values are mean of two duplicate assays carried out on different days. B) A double reciprocal plot of these data was non-linear at high substrate concentrations. C) An Eadie-Scatchard plot of estimated initial velocities over substrate concentration (V/S) against velocity (V) in rat epididymis homogenates at pH 7.0. This plot could be described by two enzyme activities, an alleged type I (—) and type II (...) 5α-reductase isozyme. The abscissa intercepts give the respective Vmax's, while the slope indicates $-Km^{-1}$.

The affinity constants of the subtype activities calculated from these plots were characteristic for the already established 5α-reductase isozymes: the mean Km was 752 nM for the alleged type I isozyme and 5.29 nM for the type II. The mean Vmax value of the type I was 50-fold higher than that of the type II isozyme: 5.78 vs. 0.113 pmol/(min*mg protein)(*table 3.1*). The efficiency ratio Vmax/Km in the rat prostate at pH 7.0 was $7.68*10^{-6}$ l/(min*mg protein) for the type I and $21.3*10^{-6}$ l/(min*mg protein) for the type II 5α-reductase subtype (*table 3.1*); the total Vmax/Km ratio for 5α-reductase activity at pH 7.0 in rat prostate homogenate was $29.0*10^{-6}$ l/(min*mg protein). Almost 75 % of this ratio was accounted for by the type II and 25 % by the type I isozyme.

Table 3.1: Enzyme characteristics of type I and II 5α-reductase in rat prostate and epididymis homogenates at pH 7.0. Values are mean of two duplicate measurements with S.E.M. (n=4) carried out on two different days.

subtype:	type I			type II		
tissue:	Vmax pmol/(min* mg protein) (S.E.M.)	Km nM (S.E.M.)	Vmax/Km *10⁻⁶ l/(min*mg protein)	Vmax pmol/(min* mg protein) (S.E.M.)	Km nM (S.E.M.)	Vmax/Km *10⁻⁶ l/(min*mg protein)
prostate	5.78 (0.09)	752 (32.5)	7.68	0.113 (0.02)	5.29 (1.85)	21.3
epididymis	0.980 (0.16)	1397 (539)	0.70	0.780 (0.03)	3.14 (0.41)	248

Figure 3.1B shows the pH-dependency of 5α-reductase activity in rat *epididymis* homogenate at a pH-range of 4.0 to 8.0 with 1 μM T as substrate. Highest enzyme activity was found at pH 5.0, indicative for the presence of type II 5α-reductase. Activity was low at neutral pH. 5α-reductase characteristics were also assessed at pH 7.0 with a substrate range of 2 nM to 3.2 μM (*figure 3.3A*). As in the rat prostate, a Lineweaver-Burk plot of the data was non-linear at high substrate concentrations (*figure 3.3B*). These data were also analyzed using an Eadie-Scatchard plot of V/S against V (*figure 3.3C*). The enzyme characteristics obtained were typical for the type I and type II 5α-reductase isozymes. The mean Km values were 1397 and 3.14 nM respectively. The Vmax values of the type I and II 5α-reductase were similar: 0.98 and 0.78 pmol/(min*mg protein) respectively (*table 3.1*). The Vmax/Km ratio was $0.70*10^{-6}$ l/(min*mg protein) for the type I and $248*10^{-6}$ l/(min*mg protein) for the type II 5α-

reductase subtype. Thus in epididymis, only about 0.3 % of the total Vmax/Km ratio at pH 7.0 was accounted for by the type I isozyme.

In figure 3.4 the Vmax of the respective subtypes at pH 7.0 were compared with the Vmax/Km efficiency ratios at this pH of both isozymes in rat prostate and epididymis. The relative amount of isozyme activity present can be measured by the specific activity, Vmax; in rat prostate (*figure 3.4A*) a 50-fold higher Vmax for the type I isozyme was found than for the type II isozyme, in the epididymis (*figure 3.4B*) the specific activities for both isozymes were almost equal. Nevertheless, measuring the potential *in vivo* activity in the prostate as reflected by the efficiency ratio, Vmax/Km (*figure 3.4A*), it appeared that isozyme type I accounts for 25 % of total enzyme activity at pH 7.0, In the epididymis (*figure 3.4B*) the type II isozyme was quantitatively responsible for 5α-reductase activity at physiological T concentrations and at neutral pH (99.7 %).

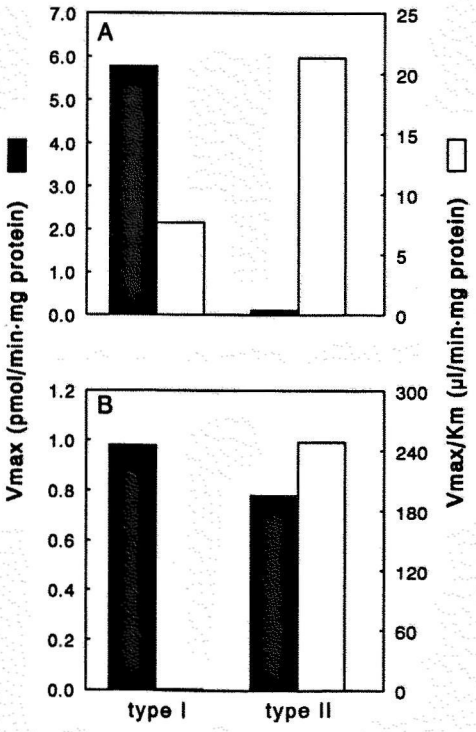

Figure 3.4: 5α-Reductase type I and type II isozyme activity in A) rat prostate and B) rat epididymis homogenates as measured by the Vmax (solid bars) or Vmax/Km ratio (open bars) at pH 7.0. Each enzyme quantification method would indicate a different isozyme activity ratio in these tissues.

3.5 DISCUSSION

The assay employed in this paper has been validated in an earlier paper [13]. The putative hysteretic behavior of the isozyme, which would extensively hamper valid measurement of enzyme activity was only observed at the acidic pH-optimum of the type II isozyme [13, 16]. With the work of Thigpen et al. in mind, we measured both isozyme activities at neutral pH, as it was reported that the acidic pH-optimum of the type II isozyme was an artifact [8]. At neutral pH, we did not observe hysteresis [13]. Therefore, the enzyme activities we measured were valid estimates of initial velocity. The affinity constants established at pH 7.0 in the present study in rat prostate (752 and 5.29 nM) and epididymis (1397 and 3.14 nM) are characteristic for the type I and type II rat and human steroid 5α-reductase subtypes reported in literature [7, 8, 13]. This strongly suggests that the non-linear plots found in rat prostate and epididymis homogenates can be attributed to these two isozyme activities. Moreover, as we measured isozyme activities at neutral pH and applied a citrate buffer, in which negative cooperativity as observed in an acetate buffer was not exhibited, the present findings did not represent the negative cooperativity of the human type II isozyme exhibited in the hyperplastic prostate at acidic pH [16].

Classically, type I or II 5α-reductase isozyme activities are detected by measuring their specific pH-optima. The presence of type II 5α-reductase can indeed be assessed by the use of a pH-profile as presented here for the rat *prostate*, where the peak at pH 5.0 indicates type II activity. The acidic pH-optimum of the type II 5α-reductase isozyme and the broad neutral pH-optimum of the type I isozyme would indicate that the maximum velocities at pH 5.0 and pH 7.0 are a measure for respectively type II and type I 5α-reductase isozyme activity. This Vmax at optimal pH has been used to characterize type I and II tissue specific distribution [17]. Using this definition, according to previous results [13] in rat prostate equal amounts of isozyme activities would be found. However, as there is now growing evidence that the type II isozyme does operate at neutral pH [8], the enzyme activity measured at acidic pH does not properly reflect true type II activity. In a tissue where considerable type I activity is present, like rat prostate [7], quantification of type II isozyme activity at neutral pH usually is hampered. In this paper a method is described by which both isozyme activities can be assessed simultaneously. The Vmax values at pH 7.0 of both isozymes were quite different from those measured at optimal pH: the results for 5α-reductase enzyme activity in rat prostate homogenate would indicate a major role for

the type I isozyme (50-fold higher Vmax). The efficiency ratios, Vmax/Km, indicate a predominant role of the type II isozyme in the rat prostate at neutral pH. The type I isozyme, however, has a ratio which is still 25 % of the total 5α-reductase efficiency ratio at pH 7.0.

The pH-profile of 5α-reductase activity in the rat *epididymis* reveals minimal activity at neutral pH and high activity at pH 5.0. The maximum activity found at pH 5.0 far exceeds that found at pH 7.0, indicating that the major amount of 5α-reductase in this tissue must be attributed to the type II isozyme. However, the activity at neutral pH can be attributed to either subtype, depending on the affinity constants at this pH. When similar amounts of both isozyme activities are present, detection of either subtype depends on the range of substrate concentrations used. At lower, physiological, concentrations a high affinity enzyme (type II 5α-reductase) will be found. At high concentrations the enzyme activity found will be largely attributable to the type I 5α-reductase. In an earlier study [13] we were not able to detect type I 5α-reductase activity in rat epididymis homogenates as we used T concentrations below 1 μM. However, incubating epididymis homogenates with higher T concentrations, we now did detect type I activity. The similar specific activities, Vmax, of type I and type II 5α-reductase at pH 7.0 indicate the presence of similar amounts of isozyme activities in this tissue. This agrees with results from immunocytochemical studies [9] and with studies in which type I and type II mRNA content is quantified in rat epididymis tissue [7, 10, 11]. However, as the efficiency ratio Vmax/Km is a better indicator of potential 5α-reductase activity [8, 15], our results indicate that at pH 7.0 only approximately 0.3 % of the total Vmax/Km ratio is accounted for by the type I isozyme. However, this type I isozyme is reportedly expressed in a small portion of the epididymis, the initial segment [9]. A highly concentrated expression in a specific segment could -notwithstanding our results presented in this paper- imply a significant contribution of this isozyme to DHT formation. Our results mark the intricacies when comparing immunocytochemical studies, 5α-reductase mRNA measurements and 5α-reductase activity measurements in rat epididymis, even more so as immunocytochemistry and mRNA measurements semi-quantitatively detect the presence of enzyme and not enzyme activity per se.

The role of the type I isozyme has been described as catabolic as it is amply found in liver tissue [7]. However, the presence of the type I 5α-reductase isozyme in the rat androgen target tissues prostate and epididymis suggests an additional role for this

subtype. On the other hand, considering the rather high affinity constant, the type I isozyme will only be active at high concentrations of T in the microenvironment of the isozyme. As all prostatic rat steroid 5α-reductase activity is reportedly located in the nucleus [18, 19, 20], one can hypothesize that the type I isozyme could therefore be active when T accumulates in the nucleus, e.g. due to binding to the androgen receptor [21, 22]. As the Vmax/Km ratio of the type I isozyme still contributes substantially to the total potential *in vivo* 5α-reductase activity, an anabolic function for this subtype in rat prostate is plausible. In rat epididymis distinct 5α-reductase activities have been detected in the nucleus and microsomes [23, 24, 25]. Immunocytochemical studies suggest a microsomal localization of the type I isozyme [9]. Accumulation of T, necessary for the type I 5α-reductase to be active in the rat epididymis, should thus occur in the cytoplasm. The role of the type I isozyme in this tissue is therefore different from that in the prostate, even more so as it is reportedly confined to the initial segment of the epididymis [9].

In summary, we present here evidence for type I 5α-reductase activity in rat epididymis homogenates in addition to the well established type II activity. The non-linear plots of 5α-reductase activity we found in rat prostate and epididymis homogenates at neutral pH could be adequately explained by the presence of two isozymes with affinity constants characteristic of the type I and type II isozymes. In rat prostate the presence of both isozymes is undisputed and this tissue can thus serve as a control for the results presented here for the rat epididymis. The results obtained for the type I activity in the rat prostate indicate a possible anabolic role for this subtype. Although the Vmax in the epididymis indicates similar amounts of type I and type II 5α-reductase activity at neutral pH, almost all potential in vivo 5α-reductase activity, as determined by the efficiency ratio Vmax/Km, is probably attributable to the type II isozyme. The highly segmental expression of the type I isozyme in the epididymis should be considered to allow for correct quantification of its in vivo contribution to DHT formation.

ACKNOWLEDGEMENT

Dr. H.A. Ross is greatly indebted for his helpful discussions on enzyme kinetic analysis.

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CHAPTER 4

KINETIC ANALYSIS OF STEROID 5α-REDUCTASE ACTIVITY AT NEUTRAL pH IN BENIGN PROSTATIC HYPERPLASTIC TISSUE: EVIDENCE FOR TYPE I ISOZYME ACTIVITY IN THE HUMAN PROSTATE

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Journal of Steroid Biochemistry and Molecular Biology 57 (1996) in press

4.1 SUMMARY

In human benign prostatic hyperplastic (BPH) tissue homogenates 5α-reduction of testosterone was examined at neutral pH. As Lineweaver-Burk and Eadie-Scatchard plots of estimated initial velocities against a wide range of substrate concentrations of 2 nM to 3.2 μM were non-linear, the existence of two 5α-reductase isozymes in this tissue was surmised. Indeed, enzyme parameters at pH 7.0 suggested the presence of two isozymes with affinity constants of 1995 nM and 11.8 nM, characteristic of the well established human steroid 5α-reductase isozymes type I and II respectively. The physiological roles of these isozyme activities remain puzzling. The specific activities, Vmax, of these subtypes indicated an approximately 6-fold higher maximum velocity of type I than of type II 5α-reductase in the human hyperplastic prostate at this pH. In contrast, the efficiency ratios, Vmax/Km, demonstrated that the type II isozyme had a nearly 27 times higher potential *in vivo* activity than the type I isozyme, and is therefore most probably quantitatively responsible for dihydrotestosterone formation at physiological testosterone levels in this tissue at neutral pH. This is the first full paper on type I 5α-reductase activity in human BPH tissue.

4.2 INTRODUCTION

Steroid 5α-reductase (E.C. 1.3.99.5) is a NADPH-dependent enzyme capable of 5α-reducing a number of steroids with a 4,5 double bond and a 3-oxo group, including glucocorticoids, progestogens, mineralocorticoids, androgens [1] and non-androgens such as the pheromone precursor 4,16-androstadien-3-one [2,3]. The 5α-reduction product of testosterone (T), dihydrotestosterone (DHT), has been implicated in the pathogenesis of benign prostatic hyperplasia (BPH) [4].

Earlier studies on the 5α-reductase enzyme characteristics in prostatic epithelium and stroma revealed a difference in affinity constants, which might indicate the existence of two isozymes in the human prostate [5,6,7,8]. As it is now well established that there are two human 5α-reductase isozymes i.e. type I and type II, with distinct pH-optima, tissue distribution, affinity constants and sensitivity to inhibitors [as reviewed in 1], one might assume that the human prostate indeed contains both of these isozymes.

Prostatic 5α-reductase type II mRNA, -protein and -activity have been detected using cDNA probes [9,10], immunoblotting and immunocytochemistry [10,11,12], and pH-profiles of 5α-reductase activity [1,13] respectively. In patients with 5α-reductase type II deficiency (male pseudohermaphroditism) prostate development is impaired, indicating the importance of this 5α-reductase isozyme for the growth and development of the human prostate [14,15]. It is therefore intelligible that a specific type II isozyme inhibitor, finasteride, has been developed for treatment of benign prostatic hyperplasia.

Data concerning the presence of the type I 5α-reductase in human prostate, however, are conflicting; the first human 5α-reductase cDNA was derived from prostatic tissue [13,16] and was shown to code for the type I isozyme. Using cDNA probes, mRNA for the type I 5α-reductase has been found in the human prostate [9], whereas other investigators failed to demonstrate type I 5α-reductase mRNA in this tissue [10]. Furthermore, immunoblotting studies using specific antibodies against the 5α-reductase protein denies type I immunoreactivity [10,12]. On the other hand, apparent affinity constants for 5α-reductase found in human prostatic tissues are consistent with type I activity [17] and preliminary data have been published in abstract form indicating both type I and type II 5α-reductase activity in this tissue [18].

There is growing evidence that both 5α-reductase isozymes operate at neutral pH [19]. By incubating homogenates of rat prostate and epididymis with a wide range of substrate concentrations, we were recently able to measure both rat isozymes simultaneously [20]. In the present study we applied the same procedure to human BPH homogenates. A careful analysis of 5α-reductase activity at pH 7.0 showed non-linear Lineweaver-Burk and Eadie-Scatchard [21] plots of initial velocity against substrate concentration. This could be adequately explained by the presence of two isozymes in BPH tissue. The kinetic characteristics of these isozymes were in concordance with those of the well established human steroid 5α-reductase type I and II isozymes. Both the type I and type II isozyme specific activities and potential *in vivo* activities were determined.

4.3 MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]T (3.74 TBq/mmol) and [1α,2α(n)-^{3}H]17ß-hydroxy-5α-androstan-3-one (DHT) (2.00 TBq/mmol) were purchased from Amersham (Amersham, U.K.). [9,11-3H]5α-androstane-3α,17ß-diol (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high performance liquid chromatography (HPLC, see below) before use. Non-labelled T was purchased from Steraloids (Wilton, NH). Diethylether (p.a.), n-hexane (LiChrosolv) and 2-propanol (LiChrosolv) were purchased from Merck (Darmstadt, FRG). All other chemicals used were of analytical grade.

Protein levels were determined by a modification of the method of Lowry et al. [22] against a standard of bovine serum albumin (OHRD 20/21, Hoechst-Behring, Marburg, FRG). The assay was modified for microtiter-plates and had a sensitivity of 25 μg per well.

Buffers

Homogenization buffer consisted of 20 mM phosphate (Merck), 1 mM monothioglycerol and 0.25 M sucrose (Merck), pH 7.0. The incubation buffer consisted of 200 mM Tris (2-amino-2-hydroxymethylpropane-1,3-diol, Merck) and citric acid monohydrate (Merck), pH 4.5-8.0 or pH 7.0, and 2 mM NADPH tetrasodium salt (Merck).

Tissue preparation

Human benign prostatic hyperplastic tissues were obtained from 15 different patients, age 53-69 years, undergoing transurethral resection. Prostatic tissue chips were placed in liquid nitrogen for transport to the laboratory. Aliquots of each specimen were sent to the Department of Pathology for histological confirmation of BPH. Tissues were kept at -80 °C or

processed immediately. All subsequent procedures were performed on ice. Tissues (9.3 gram total w.w.) were pooled, thawed, and minced with razor blades into small pieces. Minced tissue was homogenized in ice-cold homogenization buffer with a 7 ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ) with a loose and a tight fitting pestle. The homogenate was filtered twice through nylon netting of 50 and 140 mesh respectively to remove cell debris. By this procedure –without centrifugation– a full homogenate with nuclei and cytosol is obtained. The pooled BPH homogenate was diluted with homogenization buffer to 5.4 mg protein/ml.

5α-reductase assay

Radiolabelled T in ethanol was brought to final concentration (2 nM to 3.2 μM) by isotopic dilution with non-labelled steroid in Pyrex culture tubes (borosilicate glass, 12x75 mm, Corning Inc., Corning, NY). Ethanol was evaporated under a mild nitrogen stream at room temperature. Incubation buffer (800 μl) was added and the tubes were put into a shaking water bath at 37 °C at least 10 minutes before the start of the incubation to ensure the substrate was dissolved (97%). A tube with the homogenate (50-100 μl) and the appropriate amount of cofactor (NADPH, 2 mM final concentration) was diluted to 200 μl with 100-150 μl incubation buffer and kept on ice. The incubation was started by adding 200 μl of the homogenate and cofactor mixture to the pre-heated tubes with substrate. After 10-30 minutes the incubation was terminated by adding 100 μl of 3 M NaOH. This 5α-reductase assay protocol was optimized for rat tissues (prostate and epididymis) in an earlier paper [23]. The assay was evaluated for BPH tissues, and was checked for linearity in time and protein concentration. T metabolism was not allowed to exceed 15% by varying enzyme concentration and incubation time. To extract metabolites, 4 ml of ice-cold diethylether was added, and the tubes were capped and shaken. The water phase was frozen in an alcohol bath with dry-ice, the organic phase decanted and evaporated under nitrogen. Metabolites were dissolved in 100 μl hexane for HPLC.

HPLC

Metabolized steroids were separated on a Hibar LiChrosorb Diol-column (length 250 mm, 5 μm, Merck), equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC-system included a Waters 610 Fluid Unit, a Waters 600E System Controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane-propanol 96:4, v/v) was 1.5 ml/min at a pressure of 680 psi. Radioactivity was monitored with a FloOne Beta Radiomatic A500 radio-chromatography detector (Packard-Canberra Benelux, Tilburg, The Netherlands) with a 500 μl cell and a liquid scintillation flow of 1.5 ml/min (Aqua-Luma, Lumac-LSC, Olen, Belgium). The counting efficiency for tritium was 47%. The percentage formation of DHT and 5α-androstane-3α/ß,17ß-diol was used to estimate 5α-reductase activity. Overall experimental recoveries for T, DHT and 5α-androstane-3α,17ß-diol were 90-93%.

Calculation of enzyme characteristics

Velocities were plotted against T concentration, and Km and Vmax were calculated using a non-linear regression procedure based on a Michaelis-Menten equation for two isozyme activities. A double reciprocal plot of the obtained estimated initial velocities against substrate concentration was used. This Lineweaver-Burk plot appeared to be non-linear at high T concentrations. Furthermore, an Eadie-Scatchard plot of velocity over substrate concentration (V/S) against velocity (V) was used for a more even weighting of points [21]. This plot was clearly non-linear and indicated the presence of isozyme activities. Two 5α-reductase activities with different Vmax and Km could be calculated using the same non-linear regression procedure with fitting to least squares. For the use of the Vmax/Km ratio as an index of potential enzyme activity one has to consider that endogenous T concentrations are much lower than the Km of either 5α-reductase subtype [5]. Therefore applying $Km >> [S]$ in the Michaelis-Menten equation gives: $V = Vmax/Km*[S]$. So, at physiological T concentrations, the enzyme reaction velocity is proportional to Vmax/Km.

4.4 RESULTS

Validity of the 5α-reductase assay

In this study we investigated the 5α-reductase characteristics in human benign prostatic hyperplastic tissue homogenates. The 5α-reductase assay we applied was optimized and its validity checked in rat tissues as reported in an earlier paper [23]. The validity of this protocol was confirmed for the present study in BPH homogenates. In the absence of cofactor, the enzyme was unstable at 37 °C. This deterioration could be countered by adding cofactor during the preincubation. With 2 mM NADPH, the enzyme activity was stable for at least 30 minutes at 37 °C. This stabilization of the enzyme activity by preincubating with cofactor has been reported earlier by others [24, 25]. Furthermore, the initial burst in the time course of the 5α-reduction of T, reported by others in BPH particulate fractions [26] and by us in rat epididymis and prostate [23], was also countered with the applied protocol in the present study. This procedure led to a linear time course during the incubation period for at least 30 minutes, allowing for the estimation of initial velocities. Finally, 10 to 100 μl of the diluted homogenate (0.054 to 0.54 mg protein) was incubated under the established conditions mentioned in the Materials and Methods section. The velocities were linear for the entire protein concentration range tested (results not shown).

pH-optimum

A pH-profile of enzyme activity was made over the pH range 4.5 to 8.0 using 4

substrate concentrations (*figure 4.1*). For purpose of comparison, the initial velocity at the optimum pH is arbitrarily set at 100% for each of the T concentrations. At T concentrations of 100, 200 and 1000 nM, the optimum of initial velocity was found at pH 5.5. However, at a lower T concentration of 10 nM, the optimum was at pH 6.0.

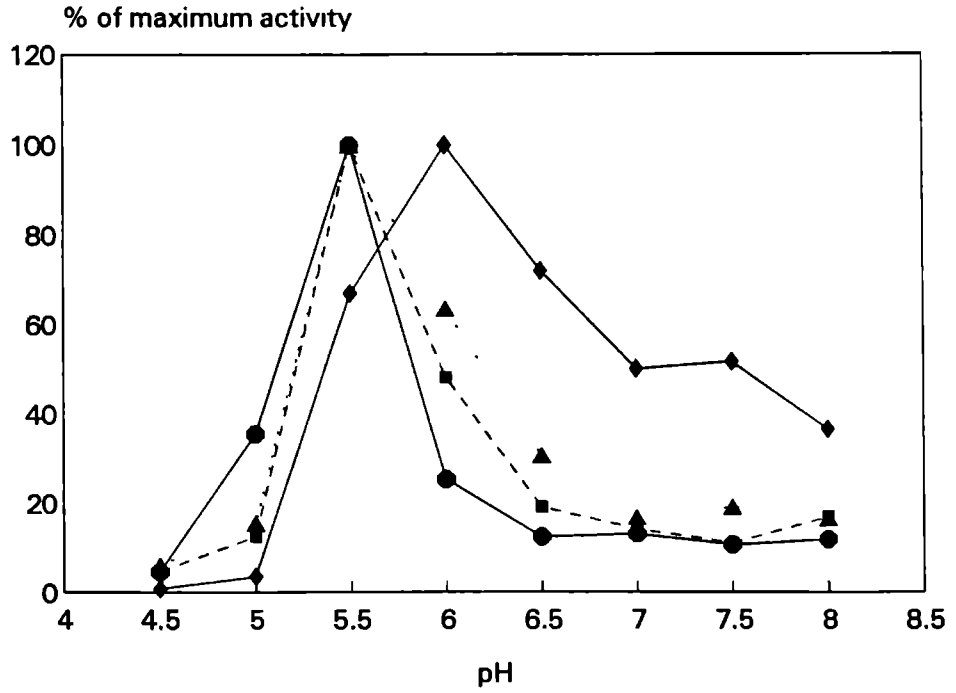

Figure 4.1: pH-Profiles of 5α-reductase activity at 2 mM NADPH and with (-◆-)10 nM, (-▲-) 100 nM,(-■-) 200 nM and (-●-) 1000 nM T as substrate. The optimum pH for 5α-reductase activity in human BPH tissue depends on the substrate concentration chosen. For purpose of comparison at each concentration the optimum activity was arbitrarily set at 100%.

Isozyme activities at neutral pH

At pH 7.0 the initial velocities were estimated over a wide concentration range of 2 nM to 3.2 μM T (*figure 4.2A*). As the double reciprocal plot of these data was non-linear at high T concentrations (*figure 4.2B*), a non-linear fit to least squares was used and the enzyme parameters of two 5α-reductase isozymes could be calculated. An Eadie-Scatchard plot of V/S against V was used for a more even weighting of points (*figure 4.2C*) and was more clearly non-linear.

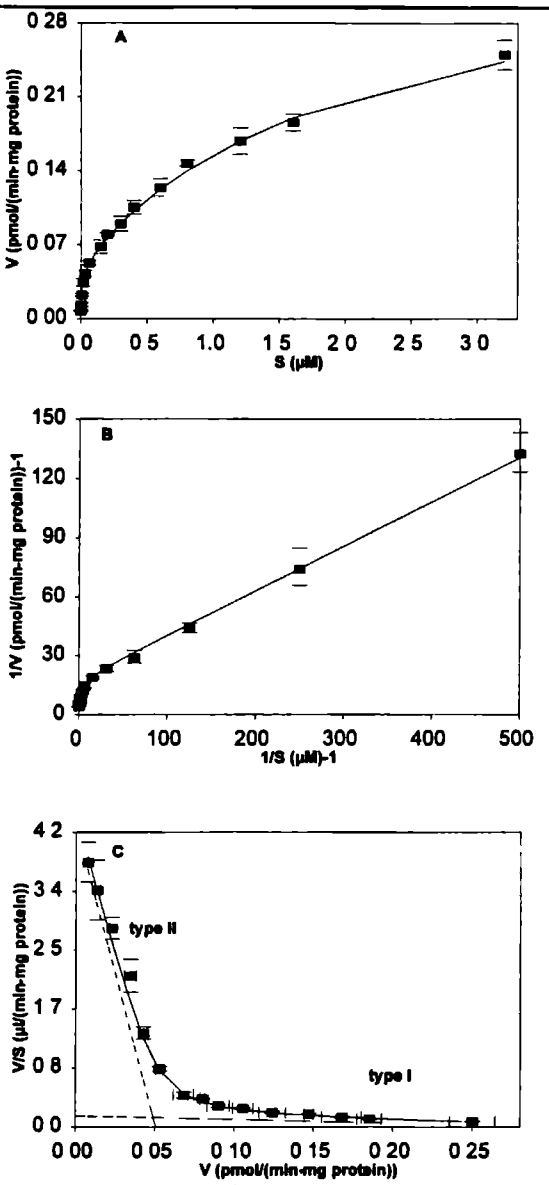

Figure 4.2: A) Estimated initial velocities (V) of 5α-reductase activity in human BPH tissue homogenates at pH 7 0 with 2 mM NADPH and with 2 nM to 3.2 µM T as substrate (S). Values are given as mean ± S D. of three duplicate assays carried out on different days. B) A double reciprocal plot of the data in figure 4.2 is non-linear at high T concentrations. C) An Eadie-Scatchard plot of the data reported in figure 4.3 of estimated initial velocities over substrate concentration (V/S) against velocity (V) in human BPH tissue homogenates at pH 7.0. This plot gives a more even weighting of points and is clearly non-linear. It could be described by two enzyme activities, an alleged type I (—) and type II (...) 5α-reductase isozyme. The abscissa intercepts of these lines give the respective Vmax's, while the slopes indicate -Km^{-1} values of the isozymes. Both the x-values and y-values are given as mean ± S.D. of three duplicate assays.

One subtype was calculated with a Vmax of 50.8 fmol/(min*mg protein) and a Km of 11.8 nM, which is characteristic of the established type II isozyme at this pH. The other subtype could be defined as having a Vmax of 313.1 fmol/(min*mg protein) and a Km of 1995 nM, corresponding to Km values reported for the type I isozyme. The efficiency ratio Vmax/Km of the alleged type I isozyme was $0.16*10^{-6}$ l/(min*mg protein), whereas the type II isozyme had an approximately 27-fold higher Vmax/Km ratio of $4.30*10^{-6}$ l/(min*mg protein). For purpose of comparison, the Vmax and the Vmax/Km ratios of both isozymes at pH 7.0 are shown in figure 4.3. Although the Vmax of the type I 5α-reductase isozyme was 6.2-fold higher than that of the type II, it yet appeared that the potential *in vivo* activity at pH 7.0 is mainly attributable to the type II isozyme, as evidenced by its much higher Vmax/Km ratio.

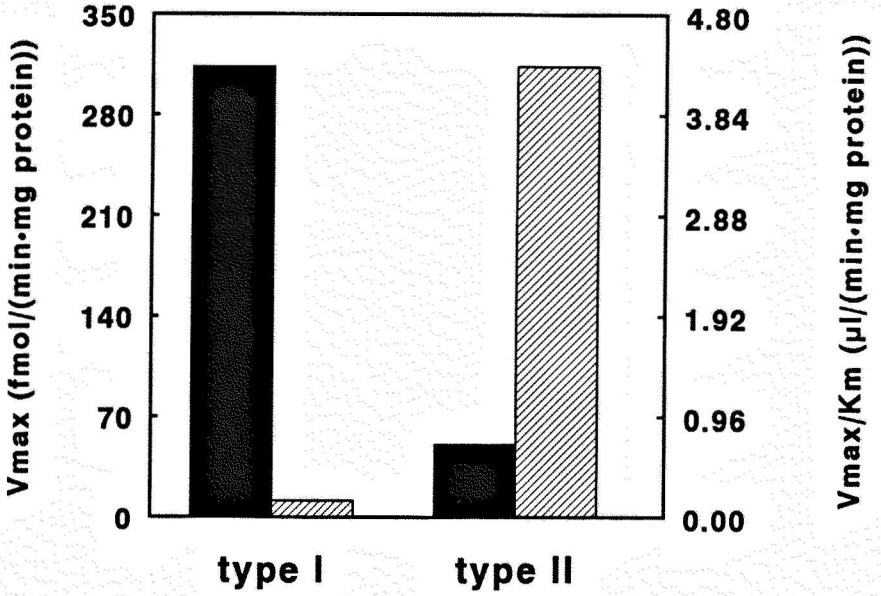

Figure 4.3: 5α-Reductase type I and type II isozyme activity in human BPH tissue homogenates at pH 7.0 as described by the Vmax (black bars) or Vmax/Km ratio (shaded bars). The specific activities, Vmax, indicate a quite different isozyme composition in this tissue than does the efficiency ratio Vmax/Km.

4.5 DISCUSSION

In this study we investigated the 5α-reductase characteristics of human benign prostatic hyperplastic tissue homogenates. In a previous paper [23] we described deterioration of enzyme activity at both pH 5.0 and at pH 7.0 in the absence of cofactor at 37 °C in rat prostate and rat epididymis. The enzyme activity was stable for 30 minutes at 37 °C with 2 mM NADPH. This also applied to 5α-reductase activities in BPH homogenates described in the present paper. The initial burst in the time course of 5α-reductase activity [23, 26] was not present with the protocol applied in this study. Furthermore, the activity was linear over the whole protein concentration range tested. Therefore, we conclude that the values we here established are reasonable estimates of initial velocity.

We established the pH-optimum for 5α-reductase activity in BPH homogenates. This pH-optimum of 5α-reductase activity in human prostate has been reported to vary from as low as pH 5.0 [13], to pH 5.5 [26] or even 7.0 [8,17]. In this study we measured the pH-dependency of 5α-reductase activity at substrate concentrations ranging from 10 to 1000 nM T. At the higher T concentrations (100 to 1000 nM), the pH-optimum was found at 5.5. At the lowest T concentration tested (10 nM), however, the optimum was at pH 6.0. Thus, the pH-optimum strongly depends on the substrate concentration chosen. As the affinity constant for 5α-reductase activity depends substantially on pH [19, 27], the pH-optimum found will greatly influence reported Km values for 5α-reductase in BPH tissue. This might -in part- explain the wide range of Km's reported for 5α-reductase activities [26]. We decided to measure enzyme activities at pH 7.0, as this is probably the physiological pH for both isozymes [19].

The existence of type II 5α-reductase in human prostatic tissue is now well accepted [9,10,11,12,13]. In patients with a type II 5α-reductase deficiency (male pseudohermaphroditism) the prostate is atrophic [14,15]. In these patients the type I isozyme is unaffected, indicating the crucial function of the type II isozyme in the development of the human prostate. Data concerning type I 5α-reductase diverge, as some authors deny the existence of the type I isozyme in the human prostate [10,12], whereas others claim its presence [9,13]. Preliminary data has been published reporting equal amounts of type I and type II activity in human prostatic tissue [18]. We describe here the presence of two isozymes in a full homogenate of BPH tissue,

by applying high T concentrations and by using an Eadie-Scatchard plot of the obtained data. This plot is particularly suitable for identifying two isozymes as it leads to a more even weighting of points as compared to the Lineweaver-Burk plot [21]. The affinity constants for these isozymes are typical for the type I and II 5α-reductase subtypes at neutral pH as reported in literature [16,19,27]. When Vmax is taken as a measure of enzyme concentration, our results imply that there is an approximately 6-fold higher amount of type I 5α-reductase than of type II at pH 7.0 in the human hyperplastic prostate. However, one has to be cautious regarding these results; to preserve all cellular components we did not centrifuge our homogenate, but filtered the preparation through nylon netting which might have led to a preferential recovery of epithelial components [7]. Stroma and epithelium do probably not exhibit the same 5α-reductase isozyme composition [5,6,7,8]. Thus a better recovery for the epithelial components in this study would have led to a difference in relative isozyme concentrations found. Furthermore, unlike in this paper, most studies use a centrifugation step in their experimental procedure to remove cell debris. However, whole nuclei will also be lost at 800 g, and as there might be a difference in intracellular localization between both 5α-reductase subtypes, more of a nuclear bound isozyme could have been detected with our protocol than in these other studies.

For enzyme quantification several definitions can be used: the maximum velocity at the optimum pH of the isozyme, the Vmax at neutral pH – as the acidic pH-optimum of the type II 5α-reductase is probably not physiological – or the efficiency ratio Vmax/Km as a measure of isozyme activity, instead of isozyme concentration. All these definitions can, and have been, used. At pH 5.5 a maximum velocity of 0.7 pmol/(min*mg protein) was found, at pH 7.0 the Vmax was 0.13 pmol/(min*mg protein), indicating a 5.4-fold higher type II than type I isozyme specific activity (results not shown). Considering our data of isozyme activity at pH 7.0, the Vmax of the type I isozyme appeared to be 6.2 times higher than that of the type II. However, the Vmax/Km ratios for both isozymes point to a 26.9-fold higher potential *in vivo* activity for the type II than the type I isozyme in human prostatic tissue at pH 7.0. When this efficiency ratio is used as a measure of isozyme activity, the results suggest that a specific type II isozyme inhibitor, like finasteride, should suffice in decreasing prostatic DHT tissue levels in the clinical management of BPH. However, it has not been unequivocally ascertained whether the premise of $Km >> [S]$ for the use of the efficiency ratio is valid in the microenvironment of the type I isozyme; human

prostatic 5α-reductase is reported to be located in the nucleus [28]. One can speculate that the type I isozyme could be active when T is accumulated in the nucleus to a higher substrate concentration, e.g. by binding to the androgen receptor. If this would be the case, the here reported substantial type I 5α-reductase activity might call for an evaluation of a combination therapy with type I and II isozyme inhibitors for BPH [15]. Experiments are underway in our laboratory to ascertain the intracellular localization of both isozymes and to check the validity of the Vmax/Km ratio. The discrepancies in isozyme specific mRNA, immunoreactivity and activity measurements in the human prostate might be attributable to differences in translation from mRNA to protein, or to differences in catalytic efficiency from protein to activity between the two isozymes.

In conclusion, we present here evidence for the presence of a substantial amount of type I 5α-reductase in human benign prostatic hyperplastic tissue homogenates in addition to the well established type II. Nevertheless, although type I has an approximately 6-fold higher specific activity than type II 5α-reductase at neutral pH, it is most probably the type II isozyme that is quantitatively responsible for the formation of DHT in the human prostate, considering the efficiency ratio Vmax/Km of the isozymes in this tissue. However, the intracellular and cell-type specific localization of both isozymes has to be considered so as to shed more light on the possible physiological roles of both isozymes in this tissue. The role of the reported prostatic type I 5α-reductase isozyme in BPH remains intriguing.

ACKNOWLEDGEMENTS

We like to thank Dr. H.A. Ross for his helpful discussions about enzyme kinetics and Dr. J. de la Rossette of the Department of Urology of the University Hospital St. Radboud for providing the BPH tissues.

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CHAPTER 5

DIFFERENTIAL SUBCELLULAR DISTRIBUTION OF RAT PROSTATIC STEROID 5α-REDUCTASE ISOZYME ACTIVITIES

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submitted

5.1 ABSTRACT

objective: The rat prostate, a classical androgen-target tissue, contains both known isozymes of steroid 5α-reductase, i.e. type I and type II. So far, the role of the type I isozyme has been proposed as catabolic. The abundant expression of type I 5α-reductase in an androgen-target tissue is therefore puzzling. Assessment of the subcellular localization of 5α-reductase isozymes in rat prostate might contribute in elucidating their possibly distinct roles.
methods: After obtaining crude subcellular fractions by differential centrifugation, both isozyme activities were measured at neutral pH by plotting according to Eadie-Scatchard. The observations were extended by assessment of pH-dependent velocity ratios and type II 5α-reductase inhibitor sensitivities in these subcellular fractions.
results: The results indicated a preferentially —although not exclusively— nuclear localization for the type I and a predominantly microsomal localization for the type II isozyme activity in the rat prostate.
conclusion: The nuclear localization of the type I isozyme seems not to concur with its proposed catabolic role.

5.2 INTRODUCTION

Steroid 5α-reductase (3-oxo-5α-steroid Δ^4-reductase, E.C. 1.3.1.22) is a membrane-bound, NADPH-dependent enzyme capable of 5α-reducing a number of steroids with a 4;5 double bond and a 3-oxo group, including androgens [1].
The enzyme 5α-reductase was first described in rat liver [2, 3], were it was considered to function as a catabolic enzyme. After 5α-reduction, steroids are susceptible to 3α- and 3ß-hydroxysteroid dehydrogenation, sulphation and glucuronylation, facilitating their excretion [1]. Later, 5α-reductase activity was also found in rat prostate [4-7], a classical androgen-target tissue. As dihydrotestosterone (DHT) accumulated in the nuclei of prostatic cells after administration of radiolabelled testosterone (T) to rats [4, 8], and as DHT is a more potent androgen than T in binding to the androgen receptor [9, 10], an anabolic role for the prostatic 5α-reductase has been suggested. To date, two subtypes of steroid 5α-reductase with specific pH-optima and inhibitor sensitivities, designated type I and type II, have been described in both human and rat [11-16]. Both the catabolic and anabolic roles proposed for 5α-reductase could be attributed to the respective isozymes, based on their tissue-specific localization —type I being found in the liver, type II being the predominant isozyme in the prostate— and based on their respective affinity constants [16].

Studies on the subcellular distribution of 5α-reductase activity in the rat prostate show a wide variety of results. Investigators found nuclear-bound rat prostatic 5α-reductase, yet with a considerable amount of enzyme activity in mitochondrial and microsomal fractions, ranging from 24 to as high as 74 % of total activity [6, 17-20]. These latter activities, however, might have arisen from nuclear contamination, as has been described for the human prostate [21]. Subsequent research focused on the characterization, purification and solubilization of the nuclear-bound prostatic 5α-reductase activity [6, 7, 17, 20, 22]. It is now recognized that the rat prostate expresses both isozymes of 5α-reductase [16]. Immunocytochemical studies established a nuclear localization of 5α-reductase immunoreactivity —probably attributable to the type I isozyme— in rat prostate epithelium [23]. To the best of our knowledge, the subcellular localization of 5α-reductase isozyme activities in rat prostate has not yet been elucidated.

In the present study, the subcellular distribution of 5α-reductase isozyme activities in the rat prostate was investigated to get better insight into the functional roles of both

isozymes. The results suggest a differential subcellular distribution of the two subtypes in this tissue, based on activity measurements, isozyme-specific pH-dependent velocity ratios, and inhibitor sensitivities of 5α-reductase activity in crude subcellular fractions.

5.3 MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]Testosterone (3.74 TBq/mmol) and [1α,2α(n)-^{3}H]17ß-hydroxy-5α-androstan-3-one (2.00 TBq/mmol) were purchased from Amersham (Amersham, U.K.). [9,11-^{3}H]5α-androstane-3α,17ß-diol (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high performance liquid chromatography (HPLC, see below) before use. Testosterone (T) was purchased from Steraloids (Wilton, NH). Diethylether (p.a.), n-hexane (LiChrosolv) and 2-propanol (LiChrosolv) were purchased from Merck (Darmstadt, FRG). All other chemicals used were of analytical grade. The specific rat 5α-reductase type II inhibitor L-685,273 (21,21-pentamethylene-4-aza-5α-pregn-1-ene-3,20-dione) was a kind gift of the Merck, Sharp and Dohme Corp. (Rahway, N.J., USA)

Buffers

The homogenization buffer consisted of 20 mM phosphate (Merck), 1 mM monothioglycerol, 50 μM NADPH tetrasodium salt (Merck) and 0.25 M sucrose (Merck) (pH 7.0). The incubation buffer consisted of 200 mM Tris (2-amino-2-hydroxymethylpropane-1,3-diol, Merck) / citric acid monohydrate (Merck) and 2 mM NADPH (pH 5.0 or pH 7.0).

Animals and tissue preparation

Ten Wistar rats from the local breeding facility of 7-13 weeks old (150-250 g) were killed by decapitation and whole prostates were removed, freed of adhering fat and placed in liquid nitrogen for transport to our laboratory. Tissues were processed on the same day. All subsequent procedures were performed on ice. Pooled tissues (2.3 g total w.w.) were minced with razor blades and homogenized in ice-cold homogenization buffer with a 7 ml Dounce tissue grinder (Kontes Glass Co., Vineland, NJ) with a loose and a tight fitting pestle. The homogenate was filtered twice through nylon netting of 50 and 140 mesh respectively to remove cell debris. This full homogenate was diluted with homogenization buffer to a concentration of 21 mg protein/ml.

Preparation of subcellular fractions

Crude subcellular fractions were obtained by differential centrifugation at 4 °C. After centrifugation at 1000 x *g* for 15 min in a Sorvall RC24 with a SM24 fixed angle rotor, a crude nuclear pellet was obtained. The resulting supernatant was centrifuged at 10000 x *g* for 20 min in the same rotor to obtain a mitochondrial pellet. The 10000 x *g* supernatant was

subsequently centrifuged at 108000 x *g* for 60 min in a MSE Prepspin 75 with a SP-2818 fixed angle rotor, resulting in a microsomal pellet and a supernatant containing the cytosol. All pellets were resuspended in homogenization buffer with a glass-glass tissue grinder (Kontes) to 4 (mitochondrial fraction), 8 (microsomal fraction) and to 9 mg protein/ml (crude nuclear fraction). Aliquots of 1 ml were stored at -80 °C and assayed within 4 weeks.
Protein levels were determined by a method modified from Lowry et al. [24] against a standard of bovine serum albumin (OHRD 20/21, Hoechst-Behring, Marburg, FRG). The assay was modified for microtiter-plates and had a sensitivity of 25 μg per well.

5α-Reductase assay
The assay has been described and validated in detail in earlier papers [25, 26]. In short, radiolabelled T was isotopically diluted to a range of 2 nM to 3 μM (final concentration, 10 duplicate points), dissolved in incubation buffer (800 μl) in Pyrex culture tubes (borosilicate glass, 12x75 mm, Corning Inc., Corning, NY) and subsequently preheated at 37 °C. For the inhibition experiments, the specific rat 5α-reductase type II inhibitor L-685,273 was added from stock solutions in ethanol (10 pM to 10 μM final concentration) simultaneously with T (30 nM final concentration). This inhibitor is type II specific with inhibition constants of 0.04 nM for type II and 2.8 nM for type I 5α-reductase respectively [16]. Ten to 100 μl of the enzyme preparation (40 μg to 2.1 mg protein) and the appropriate amount of cofactor (NADPH, 2 mM final concentration) was supplemented to 200 μl with incubation buffer and added to the tubes with substrate to start the incubation. After 30 minutes the incubation was terminated by adding 100 μl of 3 M NaOH, and metabolites were extracted with 4 ml of ice-cold diethylether. Percentile metabolism ranged from 1 to 16 %. Extracted metabolites were reconstituted in 100 μl hexane for HPLC.

HPLC
Metabolized steroids were separated on a Hibar LiChrosorb Diol-column (length 250 mm, 5 μm, Merck), equipped with a guard column (Resolve Silica, Waters Corp., Milford, MA). The HPLC-system included a Waters 610 Fluid Unit, a Waters 600E System Controller and a Waters U6K injector. The isocratic flow of the mobile phase (hexane / propanol 96:4, v/v) was 1.5 ml/min at a pressure of 680 psi. Radioactivity was monitored with a FloOne Beta Radiomatic A500 radio-chromatography detector (Packard-Canberra Benelux, Tilburg, The Netherlands) with a 500 μl cell and a liquid scintillation flow of 1.5 ml/min (Aqua-Luma, Lumac-LSC, Olen, Belgium). The counting efficiency for tritium was 47 %. The percentage formation of DHT and 5α-androstane-3α(ß),17ß-diol (Adiol) was used to estimate 5α-reductase activity.

Calculation of enzyme characteristics
Velocities were plotted against T concentrations (over 10 duplicate points), and Km's and

Vmax's were calculated using a non-linear regression procedure to least squares, based on a Michaelis-Menten equation for two independent isozyme activities, with the computer program Enzfitter. A double reciprocal plot (Lineweaver-Burk) of the obtained estimated initial velocities against substrate concentration was used. Furthermore, an Eadie-Scatchard plot of velocity over substrate concentration against velocity was also used, as this plot is reportedly best suited to detect isozyme activities [27]. IC_{50}'s were determined from plots of enzyme velocity against inhibitor concentration.

Statistical analysis

Values were obtained in at least two duplicate assays carried out on different days. Statistical analysis was performed using a Pearson correlation test (p denoted by p*) or a one-tailed t-test according to Welch (p denoted by p).

5.4 RESULTS

The validity of the 5α-reductase assay was established and reported in earlier papers [25, 26]. Initial velocity data were obtained at a substrate concentration range of 2 nM to 3 μM T (*figure 5.1A*) and plotted according to Lineweaver-Burk (*figure 5.1B*) and to Eadie-Scatchard (*figure 5.1C*). The best fit for isozyme activities are presented as solid lines in these figures. Lineweaver-Burk plots deviated from linearity only at the higher substrate concentrations. Eadie-Scatchard plots, however, were clearly non-linear and could be described by two enzyme activities as shown by two additional lines in figure 5.1C. All subcellular fractions exhibited similar non-linear plots (data not shown).

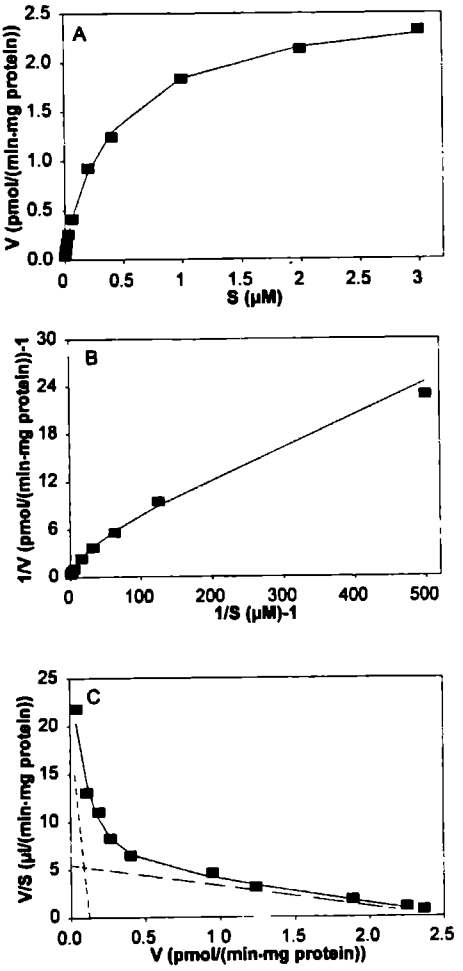

Figure 5.1: Detecting isozyme activities of rat prostatic 5α-reductase. Best fit for isozyme activity is presented by solid lines. (A) Initial velocities were obtained at 2 nM to 3 μM T and 2 mM NADPH at 37 °C in a full homogenate of rat prostate. Values are mean of at least two duplicate experiments carried out on two different days. (B) A double reciprocal (Lineweaver-Burk) plot of these data deviates from linearity only at high T concentrations. (C) An Eadie-Scatchard plot of V/S against V is clearly non-linear. Activities of type I (—) and type II (•••) 5α-reductase isozymes can be calculated from these data.

In table 5.1 data obtained in crude subcellular fractions from rat prostate are summarized. Approximately 70 % of total protein was recovered in the subcellular fractions. The total recovery of enzymatic activity was 72.4 % for type I and 73.7 % for type II 5α-reductase respectively. The yield of type I activity was highest in the nuclear fraction (*figure 5.2A*). The mitochondrial and microsomal fraction contained 3-

4 fold less type I 5α-reductase activity as compared to the nuclear fraction ($p<0.01$ and $p<0.005$ respectively). No significant differences in yield were found for the type II isozyme between the subcellular fractions, approximately 25 % of total activity being present in each of the three fractions. No 5α-reductase activity was found in the 108000 x *g* supernatant (cytosol).

Table 5.1: Protein and 5α-reductase isozyme data obtained in crude subcellular fractions of rat prostate.

subtype:	protein (mg) (%)	activity (pmol/min) (%) I	II	specific activity (pmol/min*mg protein) I	II	Km (nM) I	II
full homogenate	341.7 (100)	1010.7 (100)	48.2 (100)	2.96	0.141	528	7.2
nuclei 1000 x *g* pellet	59.2 (16.5)	472.2 (46.7)	13.4 (27.8)	7.97	0.226	613	4.9
mitochondria 10000 x *g* pellet	20.1 (5.8)	150.7 (14.9)	10.8 (22.4)	7.50	0.538	458	4.8
microsomes 108000 x *g* pellet	15.7 (4.6)	109.4 (10.8)	11.3 (23.5)	6.97	0.722	577	5.2
cytosol 108000 x *g* supernatant	149.7 (43.0)	-	-	-	-		

For both isozymes the specific activities in the subcellular fractions were all higher than in the full homogenate (*table 5.1*). The enrichment values for the isozymes, calculated as the ratio of specific activity in the subcellular fraction over that in the full homogenate, are shown in Figure 5.2B. No significant differences between the values in nuclei, mitochondria and microsomes can be seen for the type I isozyme. The enrichment for this isozyme was approximately 2.5 in all three fractions. For the type II isozyme activity, highest purification was achieved in the microsomal fraction (5 times higher specific activity), and lowest enrichment (1.6 times) in the nuclear fraction. The values for the type II isozyme in the mitochondrial and microsomal fractions were significantly higher than that in the nuclear fraction ($p<0.025$ and $p<0.005$ respectively).

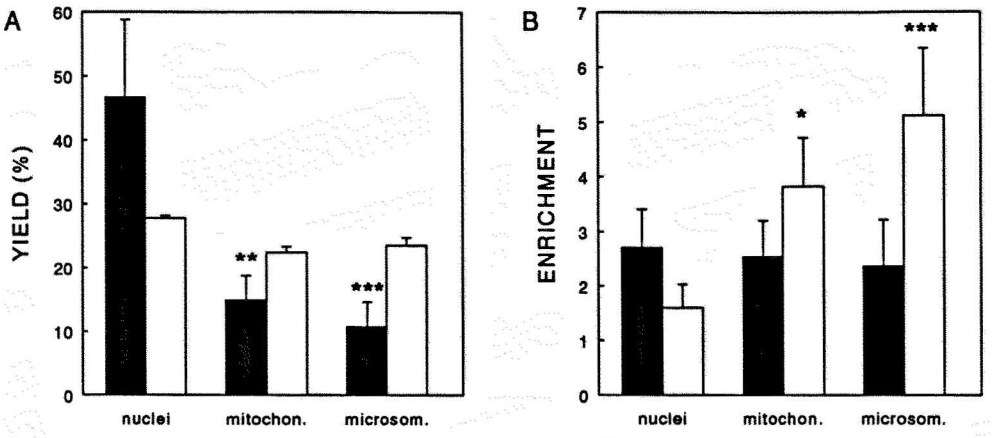

Figure 5.2: 5α-Reductase activities in crude subcellular fractions of rat prostate. Values are presented for the type I (solid bars) and type II (open bars) isozyme activities. (A) Percentile 5α-reductase isozyme activity (yield) in the different subcellular fractions. Values are mean + S.D. of at least two duplicate assays carried out on different days. The recoveries of type I activity in mitochondrial and microsomal fractions were significantly less as compared to the nuclear fraction. No significant differences between fractions were found for the type II isozyme activities. (B) Enrichment values (relative specific activities) were calculated as the ratio of specific activity in the subcellular fractions over that in the full homogenate. Values are mean + S.D. of at least two duplicate assays carried out on different days. The type II isozyme showed significantly higher purification in the mitochondrial and microsomal fractions as compared to the nuclear fraction. No significant differences between fractions were found for the type I isozyme activities. (*$p<0.025$, **$p<0.01$, ***$p<0.005$).

The affinity constants for the type I isozyme were approximately 500-600 nM in the full homogenate, as well as in the subcellular fractions (*table 5.1*). The type II isozyme activity had a Km of approximately 5 nM. These affinity constants did not differ significantly between the subcellular fractions.

To corroborate the isozyme activity assay and to further substantiate the subcellular distribution results, two other characteristics of 5α-reductase isozymes, i.e. pH-dependency of velocities and inhibitor sensitivity, were investigated in the same subcellular fractions. The ratios of Vmax values of both isozyme activities obtained with Eadie-Scatchard plots (Vmax I/II) in the subcellular fractions were correlated with isozyme-specific pH-dependent velocity ratios (v pH7.0/pH5.0) (*figure 5.3A*). The activities at pH 7.0 and pH 5.0 are generally considered indicative of type I and type II isozyme activity respectively [28]. Initial velocities were estimated with 1 μM T and 2 mM NADPH at pH 7.0 and at pH 5.0. A high positive correlation of 0.942 ($p^*<0.025$) between the ratios in the different subcellular fractions was found. The

microsomal fraction exhibited the lowest isozyme type I over type II Vmax ratio and isozyme-specific pH-dependent velocity ratio (approximately 10 and 0.32 respectively), the crude nuclear fraction on the other hand the highest values (approximately 35 and 0.78 respectively). The 10000 x *g* pellet (mitochondria) and the full homogenate exhibited intermediate values.

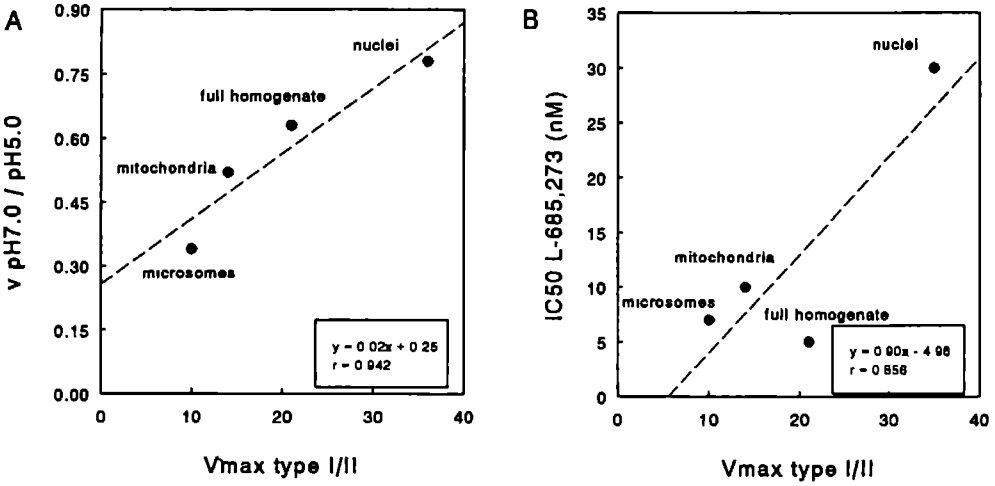

Figure 5.3: Validation of isozyme activity measurements and their subcellular distribution. (A) Initial velocities were obtained with 1 μM T and 2 mM NADPH at both pH 7.0 (≈type I) and pH 5.0 (≈type II) in subcellular fractions of rat prostate. The ratio of velocities at pH 7.0 over pH 5.0 (v pH7.0/pH5.0) were correlated with isozyme Vmax ratio values calculated as described in Figure 5.1 (Vmax I/II). A positive correlation was found of 0.942 ($p^* < 0.025$). (B) Full homogenate, crude nuclear, mitochondrial and microsomal fractions were incubated as described in the Materials and Methods section with 30 nM T and 10 pM to 10 μM of the specific rat 5α-reductase type II inhibitor L-685,273. IC_{50} values were calculated and correlated with isozyme Vmax ratio values obtained as described in figure 5.1. A positive correlation of 0.856 ($p^* < 0.05$) was found.

Because of the differences in affinity constants for T, the contribution to total 5α-reductase activity of each isozyme depends on the substrate concentration. The T concentration used for the inhibition experiments (30 nM) was chosen so that each isozyme would have an almost similar contribution to total 5α-reductase activity. This relative contribution will depend on the ratio of isozyme activities. Therefore, differences in IC_{50} values at a fixed substrate concentration can be attributed to differences in isozyme activity ratios in the subcellular fractions. Initial velocities were estimated at 10 pM to 10 μM of the specific rat 5α-reductase type II inhibitor L-685,273. IC_{50} values were 5, 10 and 7 nM for the full homogenate, mitochondrial and microsomal fractions respectively, but 30 nM for the crude nuclear fraction. As shown in Figure 5.3B the correlation between these IC_{50} values and the type I over type II Vmax ratios was statistically significant (0.856, $p^* < 0.05$).

5.5 DISCUSSION

To get better insight into the differential roles of the two hitherto known 5α-reductase isozymes expressed in the rat prostate [16], the activities of both isozymes were evaluated in crude subcellular fractions. The rat prostate has been used as a model for the human prostate because growth and development of both tissues are androgen-dependent, both contain high 5α-reductase activity, and because of the notable similarity between the human and rat 5α-reductase subtypes.

The rat and human type I 5α-reductase isozymes have been reported to have a Km for T of about 0.5 to 1 μM [16, 25, 26], whereas the type II isozymes have a Km, at neutral pH, of 4 to 50 nM [25, 26, 29, 30]. As the substrate concentration should ideally be in the neighbourhood of the Km [27], subcellular fractions of the rat prostate were incubated with a wide range of testosterone concentrations of 2 nM to 3 μM. A Lineweaver-Burk plot of these data only deviated from linearity at high substrate concentrations, whereas Eadie-Scatchard plots were clearly non-linear over essentially the whole substrate range tested.

The non-linearity of these plots might indicate negative cooperative binding of T to 5α-reductase [27]. We propose, however, that this non-linearity can better be explained by the presence of two 5α-reductase isozyme activities. The affinity constants obtained (approximately 500 and 5 nM for the alleged type I and type II activities respectively) are similar to those reported in literature for the rat and human steroid 5α-reductase isozymes at neutral pH [16, 25, 26, 29, 30]. In addition, data obtained with Eadie-Scatchard plots and isozyme-specific pH-dependent enzyme velocity ratios were closely related. Generally, the type II isozyme is considered to have an acidic pH-optimum [16]. Velocities at pH 5.0 do not properly reflect actual type II in vivo activity, as this isozyme is now considered to operate at neutral pH [30]. The ratio between activity at pH 7.0 and pH 5.0, however, does represent differences in isozyme activity ratios [28]. The correlation between data obtained by Eadie-Scatchard plots at pH 7.0 and the ratios of enzyme activity at pH 7.0 and pH 5.0 was indeed highly significant. Furthermore, the correlation between isozyme Vmax ratios and IC_{50} values of the type II-specific inhibitor L-685,273 in the subcellular fractions of the rat prostate was also significant. Altogether, these data strongly indicate the presence of two isozyme activities, resulting in the non-linear Eadie-Scatchard plots.

The subcellular localization of 5α-reductase in the rat prostate has been considered nuclear, both for enzymatic activity [6, 7, 17, 20, 22] and immunoreactivity [23]. In rat liver, type I 5α-reductase has been localized in the microsomal fraction [17], although this has been contended in a recent paper [31]. To the best of our knowledge, the subcellular localization of the isozyme activities in the rat prostate has not yet been elucidated. The results presented in this paper indicate a differential subcellular distribution of the two known subtypes of 5α-reductase in the rat prostate. Highest purification was obtained for the type II isozyme in the microsomal fraction. Furthermore, almost half of the type I activity sedimented in the 1000 x *g* pellet. However, the specific activity of the type I isozyme was similar in all three subfractions. This might be attributed to the difficulty in obtaining pure subcellular fractions due to the structural continuity between the outer nuclear membrane and the rough endoplasmatic reticulum, which has also led to controversy on the subcellular localization of the 5α-reductase type I isozyme in rat liver [31]. Though the present study does not overcome the contamination problems associated with differential centrifugation as reported for the human prostate [21], either isozyme activity can serve as a marker for the other. If the isozyme activity ratio differs between subcellular fractions, as indeed is described here, a differential subcellular distribution can be surmised. Furthermore, the results on pH-dependent velocity ratios and inhibitor sensitivities in the crude subcellular fractions confirmed our conclusion on the subcellular distribution of isozyme activities. Surprisingly, it was the type I isozyme that appeared predominantly —although not exclusively— nuclear-bound, whereas the type II isozyme had a more microsomal localization.

DHT formed by the nuclear type I isozyme will more likely bind to the androgen receptor, also located in the nucleus [32, 33], than that formed by the microsomal type II isozyme. This suggests an *anabolic* role for the 5α-reductase type I in this tissue. As in the rat prostate a high amount of 3α/ß-hydroxysteroid dehydrogenase (HSOR) activity is present in the cytoplasm [34], any DHT formed by the type II in the endoplasmatic reticulum would instantly be degraded to Adiol. Therefore, our results unexpectedly suggested a possibly *catabolic* role for the type II isozyme in the rat prostate.

However, in hypothesizing about the function of the 5α-reductase isozymes, their cell-type specific localization should also be considered. Type I 5α-reductase is expressed in the epithelial cells of the rat prostate [23, 35]. In the DHT-dependent epithelium

[36], nuclear-bound 5α-reductase type I [this study] would metabolize T to DHT to bind to the androgen receptor, suggesting a role of this subtype in the *intracrinology* of this tissue [42]. On the other hand, DHT formed by the type II in the endoplasmatic reticulum [this study] of rat prostatic stromal cells [35] is extensively metabolized to Adiol [34], which has been shown to induce BPH in the dog [39]. Stromal signals act on the epithelium in the development of certain glands [37, 38]. DHT and Adiol formed by the type II isozyme, could therefore act as *paracrine* factors in this tissue, influencing epithelial cell fate.

Finally, another possible distinction in the roles of 5α-reductase might be considered. The high affinity of the type II 5α-reductase isozyme makes it well suited for metabolizing the low concentrations of T in fetal life [30], leading to the hypothesis that it is the type II isozyme that is responsible for the *development* of the prostate. At the higher T concentrations in adulthood, type I 5α-reductase might also contribute to DHT formation, and therefore be responsible for *growth* and *maintenance* of the prostate [35]. Because of the poor development of prostates in patients with 5α-reductase type II deficiency (pseudohermaphroditism) [40], a type II inhibitor (Finasteride, Proscar®) has been developed for treatment of patients with BPH symptoms [41]. Recently, in addition to type II, we reported type I 5α-reductase activity in the human BPH prostate [26], necessitating research into this subtype and into its possible involvement in the pathogenesis of BPH.

In conclusion, Eadie-Scatchard plots of 5α-reductase activities, pH-dependent isozyme-specific velocity ratios and inhibitor sensitivities reported in this paper indicate a differential distribution of isozyme activities, i.e. a nuclear localization of the type I and a microsomal localization of the type II 5α-reductase isozyme in the rat prostate. To elucidate their roles, the cell-type specific localization of isozyme activities and the expression during ontogeny should also be taken into account.

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CHAPTER 6

3α-HYDROXYSTEROID OXIDOREDUCTASE ACTIVITIES IN DIHYDROTESTOSTERONE DEGRADATION AND BACK-FORMATION IN RAT PROSTATE AND EPIDIDYMIS

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submitted

6.1 SUMMARY

The metabolism of dihydrotestosterone (DHT) and 5α-androstane-3α,17ß-diol (3α-Adiol) was assessed in full homogenates of rat prostate and epididymis. The major degradational route of DHT was catalysed by the enzyme(s) 3α-hydroxysteroid oxidoreductase (HSOR). Enzyme kinetic characteristics Vmax, Km and Vmax/Km ratio, were obtained for the NADP(H)- and NAD(H)-dependent interconversion of DHT and 3α-Adiol at pH 7.0 and at saturated cofactor concentration. For both the reduction of DHT and the oxidation of 3α-Adiol, NAD(H) was the preferred cofactor when activities were rated by their Vmax and Vmax/Km ratio. Combining the data with the earlier established Vmax/Km ratios for the 5α-reductase isozyme type I and II activities in rat prostate and epididymis indicated that DHT —at saturated cofactor concentrations— would not be sustained in either tissue considering the reported enzyme characteristics. The reported exclusive bioavailability of the cofactors NADPH and NAD^+ *in vivo*, however, will direct the metabolic pathways in these tissues to sustain formation of DHT.

6.2 INTRODUCTION

Growth and development of the rat prostate are androgen-dependent, dihydrotestosterone (DHT) being the most active androgen. Inhibition of the formation of DHT in the rat leads to apoptosis and cell death of prostatic cells [1]. This steroid is derived from testosterone (T) through 5α-reduction by the NADPH-dependent, membrane-bound 5α-reductase (E.C. 1.3.99.5) of which two isozymes exist in both rat and human [2]. The rat and human isozymes share similar pH-profiles and affinity constants for several substrates [3, 4]. Earlier, our laboratory reported the quantification of the type I and type II 5α-reductase by enzyme activity studies in rat prostate and epididymis [Span et al. submitted].

DHT is metabolized by several enzymes [5, 6], the predominant DHT degrading enzyme in both rat prostate and epididymis being cytoplasmatic [7, 8] and/or nuclear [9] 3α-hydroxysteroid oxidoreductase (3α-HSOR, E.C. 1.1.1.50). In most male accessory sex tissues, 3ß-HSOR (E.C. 1.1.1.51) only accounts for minor DHT metabolism [6, 10, 18]. 3α-HSOR is capable of both reducing DHT ($HSOR_{red}$) to 5α-androstane-3α,17ß-diol (3α-Adiol) and back-oxidizing 3α-Adiol to DHT ($HSOR_{ox}$). Rat prostatic [11] and epididymal [12] HSOR enzymes are considered optimally active at neutral pH, like both 5α-reductase subtypes [13].

In the present paper, the NADP(H)- and NAD(H)-dependent HSOR activities were quantified at neutral pH in rat prostate and epididymis tissue homogenates, both for the reduction of DHT to 3α/ß-Adiol and the back-oxidation of 3α-Adiol to DHT, to get more insight into the role of these enzymes in the degradation and back-formation of the active androgen DHT.

6.3 MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H]Testosterone (3.74 TBq/mmol) and [1α,2α(n)-^{3}H]17ß-hydroxy-5α-androstan-3-one (dihydrotestosterone, DHT) (2.00 TBq/mmol) were purchased from Amersham (Amersham, U.K.). [9,11-3H]5α-androstane-3α,17ß-diol (Adiol) (1.48 TBq/mmol) was obtained from Du Pont-New England Nuclear (Boston, MA). All radiolabelled steroids were purified by high performance liquid chromatography (HPLC) before use. Testosterone was purchased from Steraloids (Wilton, NH). Diethylether (p.a.), n-hexane (LiChrosolv) and 2-propanol (LiChrosolv) from Merck (Darmstadt, FRG). All other chemicals used were of analytical grade.

Tissue preparation

Prostate and epididymis tissues were obtained from Wistar rats of 7-13 weeks old and processed as described earlier [14, 15]. In short, tissues were homogenized in a 20 mM phosphate buffer containing 1 mM monothioglycerol and 0.25 M sucrose using a Dounce tissue grinder. The homogenate was filtered through nylon netting of 50 and 140 mesh to remove cell debris. The resulting full homogenate was used in all further experiments.

Enzyme assays and HPLC

The HSOR enzyme assays and subsequent separation of metabolites on HPLC were modified from the assay for 5α-reductase described earlier [14, 15]. In short, tritiated substrate steroids (either ^{3}H-DHT for $HSOR_{red}$ or ^{3}H-3α-Adiol for $HSOR_{ox}$) were isotopically diluted to the desired concentration with unlabelled steroid and incubated at 37 °C in a 200 mM Tris-citrate buffer pH 7.0 with either 2 mM NADPH or NADH for $HSOR_{red}$, or with either $NADP^+$ or NAD^+ for $HSOR_{ox}$, in a final volume of 1 ml. The metabolites formed were extracted with diethylether and dissolved in 100 μl hexane for separation on HPLC. A Hibar LiChrosorb Diol column was used with an isocratic flow of 1.5 ml/min of hexane-propanol 96:4 (v/v). Radioactivity was monitored using a FloOne Beta Radiomatic A500 radio-chromatography detector. The percentile formation of either DHT or 3α/ß-Adiol was measured to estimate initial velocities.

Calculation of enzyme characteristics

Initial velocities were estimated against a substrate concentration range of 50 nM to 3.2 μM, and Km and Vmax were calculated by computerized fitting (Enzfitter program) of the data using a nonlinear regression procedure based on the Michaelis-Menten equation. Eadie-Scatchard plots of obtained initial velocity estimates were applied [15]. The Vmax/Km ratio, used in this paper, can be considered a measure of enzyme activity at low (physiological) substrate concentrations, as at $[S] << Km$, the Michaelis-Menten equation can be simplified to $v = Vmax/Km * [S]$.

6.4 RESULTS

Maximum velocity (Vmax) and apparent affinity constant (Km) values for HSOR activities were established from Eadie-Scatchard plots of the obtained estimated initial velocities in rat prostate and epididymis homogenates (*figure 6.1*). These values were obtained for the reduction of DHT to 3α/ß-Adiol ($HSOR_{red}$), as well as for the back conversion of 3α-Adiol to DHT ($HSOR_{ox}$). Results were obtained at pH 7.0 for both NAD(H)- and NADP(H)-dependent metabolism at saturated (2 mM) cofactor concentrations.

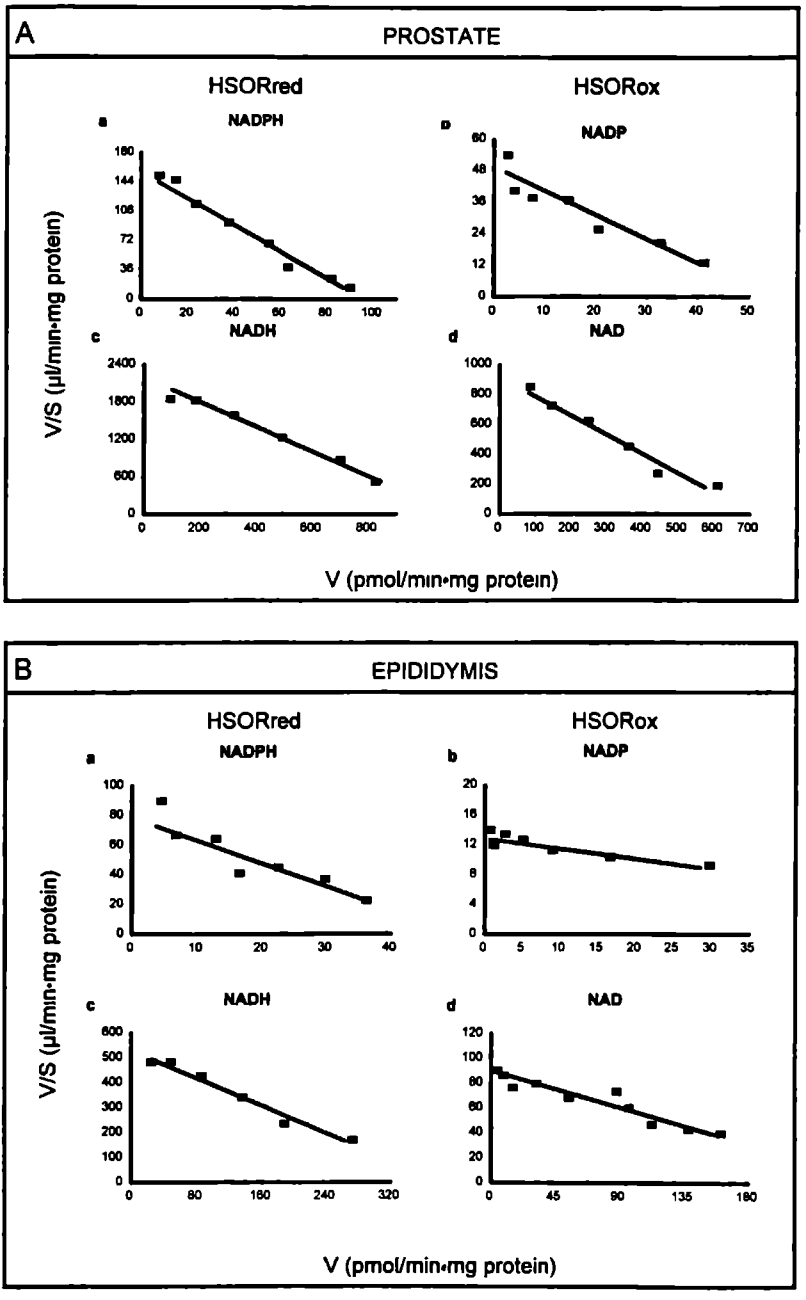

Figure 6.1: Eadie-Scatchard plots of HSOR activities obtained in A) rat prostate and B) rat epididymis homogenates. Initial velocities were estimated for the reduction of DHT to Adiol ($HSOR_{red}$) (subfigures a and c) and the oxidative back-formation of DHT from 3α-Adiol ($HSOR_{ox}$) (subfigures b and d). Both the NADP(H)-(subfigures a and b) and NAD(H)-dependent (subfigures c and d) activities were assessed. In this plot, the abscissa intercept denotes the maximum velocity, Vmax, whereas the ordinate intercept denotes potential *in vivo* activity, Vmax/Km. The slope in this plot equals -1/Km.

For each of the NADP(H)- or the NAD(H)-dependent activities, the Vmax values were approximately similar for both the reductive and oxidative pathways of HSOR in rat prostate as well as in epididymis (*table 6.1*). The Vmax values for NADH-dependent $HSOR_{red}$ were about ten-fold higher than the NADPH-dependent reductions in both tissues. The Vmax for NAD^+-dependent $HSOR_{ox}$ was also more than ten-fold higher than the Vmax for $NADP^+$-dependent oxidation in the rat prostate. In the rat epididymis, however, the Vmax ratio of $NADP^+$- and NAD^+-dependent oxidation was only a factor three. The affinity constants for the NADPH- and NADH-dependent $HSOR_{red}$ activities in both tissues were about 0.5 to 0.7 μM. The Km's for $HSOR_{ox}$ were higher (approximately 0.8 to 7.3 μM).

Table 6.1: Vmax and Km values for $HSOR_{red}$ and $HSOR_{ox}$ in rat prostate and epididymis. Both the NADP(H)- and NAD(H)-dependent activity kinetic characteristics were determined in homogenates at pH 7.0 as also shown in figure 6.1.

tissue	cofactor	$HSOR_{red}$		$HSOR_{ox}$	
		Vmax pmol/(min*mg protein)	Km nM	Vmax pmol/(min*mg protein)	Km nM
prostate	NADP(H)	96	614	54	1090
	NAD(H)	1110	505	716	781
epididymis	NADP(H)	51	645	94	7331
	NAD(H)	384	726	275	3042

The efficiency ratios Vmax/Km were established from the ordinate intercepts of the Eadie-Scatchard plots (*table 6.2*). In both rat tissue homogenates the NAD(H)-dependent HSOR efficiency ratios were manyfold (7-18 times) higher than the NADP(H)-dependent activities at low substrate concentrations. In an earlier paper the Vmax/Km ratios for 5α-reductase isozyme type I and type II activities in rat prostate and epididymis were described [Span et al. submitted]. Total 5α-reductase (type I and type II) Vmax/Km values are presented in table 6.2 for purpose of comparison. 5α-reductase activity is completely dependent on NADPH as cofactor.

Table 6.2: Vmax/Km ratios (*µl*/(min*mg protein)) for rat prostate and epididymis homogenates of the enzymes 3*α*-hydroxysteroid-oxidoreductase, the reductive ($HSOR_{red}$) and oxidative ($HSOR_{ox}$) pathway, and 5*α*-reductase type I and II [from Span et al. submitted].

tissue	cofactor	$HSOR_{red}$	$HSOR_{ox}$	5*α*-reductase
prostate	NADP(H)	156	50	29
	NAD(H)	2198	917	—
epididymis	NADP(H)	79	13	263
	NAD(H)	529	90	—

Of the enzyme activities we have measured in the rat prostate and epididymis, both 5*α*-reductase isozymes and $HSOR_{ox}$ contribute to the formation of DHT, while $HSOR_{red}$ degrades DHT to Adiol. Comparison of the Vmax/Km values of 5*α*-reductase and $HSOR_{ox}$ with $HSOR_{red}$ in table 6.2 approximates relative DHT-forming and degrading activities at low substrate concentrations. Total $HSOR_{red}$-activity (NADPH- and NADH-dependent) exceeds 5*α*-reductase activity by more than 80-fold in rat prostate. In rat epididymis, the total 5*α*-reductase activity found was much higher than in the prostate and total $HSOR_{red}$ potential *in vivo* activity exceeded total 5*α*-reductase activity only approximately by a factor two in this tissue. The ratio of the metabolism of DHT to Adiol ($HSOR_{red}$) over the back-conversion from 3*α*-Adiol to DHT ($HSOR_{ox}$) was approximately 2.4 and 5.9 in rat prostate and epididymis respectively. Total potential *in vivo* DHT-degrading activity (Vmax/Km of $HSOR_{red}$) was 2354 and 608 *µl*/(min*mg protein) in rat prostate and epididymis respectively, whereas the total Vmax/Km values of DHT-forming activities (total 5*α*-reductase and $HSOR_{ox}$) were 996 and 366 *µl*/(min*mg protein) respectively (*figure 6.2A and 6.2C*). So, at pH 7.0, the potential *in vivo* activity —as measured by the Vmax/Km ratio— of degradation of DHT to Adiol predominated over the formation of DHT from T and the oxidative back-conversion from 3*α*-Adiol at saturated (2 mM) cofactor concentrations in both tissue homogenates (*figure 6.2*). However, when only the NADPH- and NAD^+-dependent activities were compared, considering the exclusive bioavailablity of these cofactors *in vivo* (*vide infra*), DHT accumulation would be sustained in both tissues (*figure 6.2B and 6.2D*).

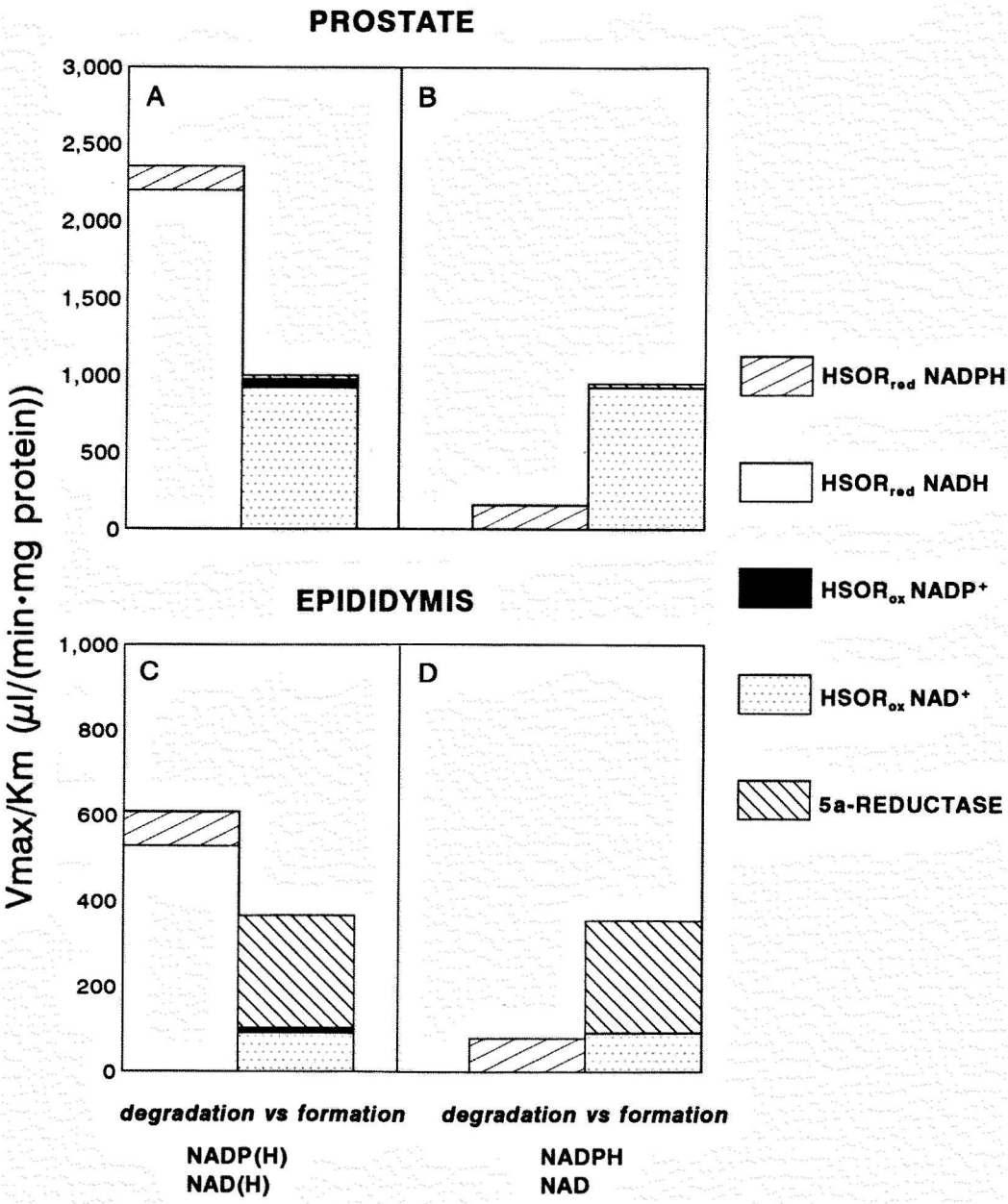

Figure 6.2: Potential *in vivo* activities (Vmax/Km in µl/(min*mg protein)) obtained in rat prostate (A and B) and in rat epididymis (C and D) homogenates. Left bars in each subfigure denote DHT-degrading enzyme activities (HSOR$_{red}$), right bars denote DHT-forming activities (total 5α-RED and HSOR$_{ox}$). A and C: All enzyme activities (NADP(H)- and NAD(H)-dependent) reported in this paper obtained as described in Materials and Methods section. B and D: NADPH- and NAD$^+$-dependent enzyme activities, thus DHT metabolism at *'in vivo'* cellular cofactor concentrations: NADPH > > NADP$^+$ and NAD$^+$ > > NADH.

6.5 DISCUSSION

In this study the reduction of DHT to Adiol and the back-oxidation of 3α-Adiol to DHT was investigated in rat prostate and epididymis homogenates. These steps in testosterone metabolism are performed by multiple isozymes of 3α- and 3ß-hydroxysteroid-oxidoreductase (HSOR). There are ample reports about the presence and cofactor-dependency of these enzymes in both rat prostate [6, 9, 16, 17, 18] and epididymis [8, 12, 19, 20].

In our hands, formation of 3ß-Adiol was not detected, but it could not be unequivocally excluded that 3α- and 3ß-Adiol epimeres were not separated with the applied HPLC system. However, in all experiments only a single Adiol peak was detected which eluted with the same retention time as tritiated 3α-Adiol. When the mobile phase was changed to hexane/propanol 98:2 v/v, thereby more than doubling the retention times, still no additional peaks could be detected. Furthermore, the affinity constants found in the rat prostate and epididymis homogenate for $HSOR_{red}$ (about 0.5 to 0.7 μM) and $HSOR_{ox}$ (0.8 to 7.3 μM) were comparable to those found in literature for 3α-HSOR activity [7, 9, 16, 17]. The affinity constant reported for rat prostatic 3ß-HSOR activity, 25.4 μM [16], is quite different from the Km's of $HSOR_{red}$ activity ascertained in the present study, indicating that most probably only 3α-HSOR activity was measured. According to literature, in most male accessory sex tissues, including the rat prostate, 3ß-HSOR only accounts for minor DHT metabolism [6, 10, 18]. 3ß-$HSOR_{red}$ activity is reportedly mainly located in the dorsal and lateral prostate and is not detectable in the ventral lobe [6, 16, 18]. In total rat prostate homogenate 3ß-HSOR activity might thus have been under the detection limit of our assay. Finally, as the Eadie-Scatchard plots of HSOR activities were basically linear, the conclusion seems to be justified that we have measured a single enzyme activity, most probably 3α-HSOR. The $HSOR_{ox}$ activities were measured with tritiated 3α-Adiol as substrate and are therefore attributable only to 3α-$HSOR_{ox}$.

In the present study, an about ten-fold higher Vmax was ascertained for the NAD(H)-dependent activities of both $HSOR_{red}$ and $HSOR_{ox}$ than for the NADP(H)-dependent activities. The NAD^+-dependent $HSOR_{ox}$ activity in the rat epididymis, however, was only three times the value of the $NADP^+$-dependent $HSOR_{ox}$. These data are seemingly at variance with some earlier reports that indicated NADP(H)-dependent HSOR activity to be greater than the NAD(H) activity in rat prostatic cytosol [7, 11].

However, when only the ventral prostate was assessed (800x*g* supernatant), the NAD(H)-dependent HSOR activity was found to exceed the NADP(H)-dependent activity [6, 18]. Furthermore, in rat prostate a nuclear-bound NADH-dependent $HSOR_{red}$ activity has been reported, being 20-fold higher than NADPH-dependent activity [9]. Two distinct 3α-HSOR activities have been found in rat pituitary, one cytosolic preferring NADPH, another membrane-bound with NADH as cofactor [21, 22]. As in the present study a full homogenate was used, differences between our study and those in literature might be due to the experimental protocol applied: unlike in our study, in many others cytosolic fractions were used or homogenates were centrifuged at 800x*g*, thereby leading to loss of any nuclear- or membrane-bound NADH-dependent HSOR activity.

$HSOR_{ox}$ activity has been reported to exceed that of $HSOR_{red}$ both for their maximum velocities in the ventral prostate [6], as for enzyme activities in cell-cultures at low substrate concentrations (50 nM) [5]. Unlike in the former study, the Vmax values for both $HSOR_{red}$ and $HSOR_{ox}$ activities reported in the present paper for whole prostate homogenates were approximately similar, which is more in line with recent data reported for the ventral prostate [18]. However, due to the lower affinity for the back conversion of 3α-Adiol to DHT, the total enzyme efficiency ratios (Vmax/Km) reported here favoured degradation of DHT at low (i.e. physiological) substrate concentrations. The activities at these low substrate concentrations as reported by Orlowski & Clark [5] should be comparable with our Vmax/Km ratios, as this ratio indicates enzyme activity at $[S] << Km$ [23]. However, in the study of Orlowski and Clark a cell culture was used where no cofactor was added, whereas our results were obtained at saturating cofactor concentrations. The difference between our study and theirs might in fact be attributable to limited bioavailability of cofactor in mammalian cells [24, 25] (*vide infra*).

The results presented in this paper do not favour accumulation of DHT in rat prostate. The high $HSOR_{red}$ Vmax/Km ratios indicated a higher tissular 3α-Adiol concentration under steady-state conditions (figure 6.2A). The Vmax/Km ratios for $HSOR_{red}$, $HSOR_{ox}$ and total 5α-reductase obtained in the rat epididymis also do not favour accumulation of DHT (figure 6.2C). Reportedly, however, tissular cofactor concentrations *in vivo* are $NADPH >> NADP^+$ and $NAD^+ >> NADH$ [24, 25]. These relative concentrations would therefore favour the reduction of DHT by the NADPH-dependent $HSOR_{red}$ and the back-oxidation by the NAD^+-dependent $HSOR_{ox}$. This profoundly changes the

interpretation of our results (figure 6.2). As the NAD(H)-dependent HSOR activities were much higher than the NADP(H)-dependent activities, the *in vivo* cellular cofactor concentrations would favour DHT formation, in sharp contrast to results obtained at saturated cofactor concentrations *in vitro*. Early reports about the *in vivo* injection of tritiated androgens indeed showed an accumulation of ^{3}H-DHT in the rat prostate [26, 27, 28], but an accumulation of tritiated 3α-Adiol in the epididymis [28]. The latter observation might indicate that the tissular cofactor concentrations in rat epididymis are different from those in the prostate and from those generally assumed *in vivo*. These results indicate that a small amount of NADH will lead to extensive degradation of DHT, because of the high NADH-dependent $HSOR_{red}$ capacity of these tissues. Thus, NAD(H) may serve as a potent regulatory factor in the degradation and back-formation of DHT in these rat tissues, as has been proposed for NADPH in the human (hyperplastic) prostate [29].

In summary, in this paper reductive and oxidative 3α-HSOR activities have been described in rat prostate and epididymis homogenates. Both the NADP(H)- and NAD(H)-dependent HSOR activities were assessed. Comparison of the enzyme activities involved in formation and degradation of DHT in these tissues, indicated that DHT concentration would not be sustained in either tissue at saturated cofactor concentrations. Exclusive bioavailability of the cofactors NADPH and NAD^+ in intact cells in vivo, however, would direct the metabolic pathways in these tissues to formation of DHT.

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CHAPTER 7
GENERAL DISCUSSION

7.1 5α-REDUCTASE ISOZYME ACTIVITIES: PITFALLS AND CONSIDERATIONS

In many androgen-target tissues, testosterone is converted to the much stronger androgen DHT, and can thus be regarded a prehormone. The enzyme that catalyses this conversion, 5α-reductase, exists as two isozymes. 5α-Reductase isozyme activities exhibit a number of peculiar characteristics *in vitro* that hamper the correct apprehension of their function *in vivo*. In this section, a few of these pitfalls in determining enzymatic activities are discussed in light of the results described in this thesis and in literature. A more precise understanding of these kinetic features of the isozyme activities obtained in tissue homogenates will aid in better appreciating the results.

7.1.1 *Enzyme stability and membrane environment*

In *chapter 2*, the instability of both type I and II 5α-reductase isozyme activities in rat prostate and epididymis homogenates was described. The deterioration of enzyme activity at 37 °C could neither be countered by anti-oxidants or protease-inhibitors, nor by EDTA or ATP. The addition of saturating amounts of the required cofactor NADPH, during the homogenization- and preincubation-procedure, however, led to stabilization of enzyme activity. This stabilizing effect of NADPH prebinding has earlier been described for human and dog prostatic 5α-reductase preparations, but denied for the rat prostatic enzyme [69]. The results described in this chapter indicate that this instability of enzyme activity is also a matter of concern in the assay for the *rat* isozymes. In the disorder of male pseudohermaphroditism due to type II 5α-reductase deficiency, mutations that altered the enzymes cofactor affinity, often also lead to instable enzymes [51, 66, 93, 116]. This led authors to propose a role for the cofactor in regulating enzyme turnover *in vivo* [116] (*section 1.6.5.3*). The mechanism by which this observed stabilizing effect of prebinding with NADPH is exerted is still unknown. The cofactor is able to bind to the enzyme before binding of the steroidal substrate [10, 46, 67]. The multitude of reports on problems in solubilization of the enzyme and on the membrane-dependency of 5α-reductase activity [15, 26, 30, 45, 57, 67, 70, 77, 78, 94] would suggest a putative susceptibility of the enzyme

conformation to membrane perturbations during the homogenization procedure (*section 1.6.5.2*). Prebinding of the cofactor might make the enzyme less susceptible to these membrane perturbations, leaving the enzyme receptive for testosterone binding and subsequent metabolism. The three-dimensional conformation of the protein is undoubtedly crucial in the enzymes activity, considering the mutational analysis of the type II isozyme that implicates both ends of the isozyme in steroid binding and exon 2 to 5 in cofactor binding [54, 92, 93] (*sections 1.4 and 1.5*).

The membrane-dependency of 5α-reductase enzymatic activity is also supported by the apparent correlation between the polarity of steroids and their Km for 5α-reductase. The hydrophobic steroid progesterone has the lowest Km for both human and rat isozymes, whereas the more polar corticosteroids have the highest Km's [3, 82, 109]. 5α-Androsta-5,16-dien-3-one, the extremely apolar pheromone-precursor formed in the human testis [115], has an apparent affinity constant for rat epididymal 5α-reductase equivalent to progesterone (*unpublished observation*). Progesterone has been reported to induce membrane-perturbations and alter the fluidity of artificial membranes and of spermatozoal membranes [100], which indicates that this steroid does indeed freely enter the membrane, and —by changing membrane fluidity— may influence enzyme activity. This process, i.e. inserting into the phospholipid bilayer membranes and subsequently altering its fluidity, is in fact one of the putative mechanisms whereby steroid hormones mediate rapid effects [11, 117] that can not be explained by the classical mechanism of androgen action mediated by the androgen receptor [14, 84, 85]. Mutations that alter the substrate binding [92, 93] and the tetrapeptide segment responsible for the isozymes sensitivity to finasteride [110] lie in *hydrophobic* portions of the enzyme in the exon 1 and exon 4 region (*figure 1.2*), suggesting that the substrate must enter the membrane in order to bind to the enzyme. Furthermore, phospholipases C and A2 decreased the affinity of testosterone for rat epididymal 5α-reductase [15]. Therefore, it seems that the affinities for steroid substrates are modulated —at least to a certain extent— by the membrane environment of the enzyme.

In contrast to the steroid binding domain, the binding domain for the (polar) cofactor NADPH [8] lies within a *hydrophilic* portion of the protein in the exon 3 region (*figure 1.2*) [2]. It has been established that the carboxyl terminus of 5α-reductase is located on the cytoplasmatic side of the endoplasmatic reticulum of transfected CHO cells [109]. When these cells were gently permeabilized with digitonin —resulting in

broken cell membranes, but leaving the endoplasmatic reticulum (the subcellular compartment of 5α-reductase isozymes in these cells) intact— NADPH addition was required to sustain enzyme activity [109]. Therefore, the conclusion seems justified that the hydrophilic cofactor binding domain of the enzyme is located such that cytoplasmatic NADPH has easy access. Contrary to steroid-binding, binding of the cofactor is thus not regulated by the membrane-microenvironment of the enzyme. One might speculate on a chain of events during homogenization of the tissue (perturbations or folding of the membrane environment and thereby inducing an alteration in the enzyme conformation, or possibly the formation of 'inside-out' membrane vesicles, thereby secluding the cofactor binding site), leading to progressive difficulty in binding the cofactor to its hydrophilic binding site. This would ensue in loss of enzyme activity, which might only be countered by prebinding of the cofactor before these events irreversibly have taken place.

7.1.2 *Hysteresis and cooperativity*

In *chapter 2*, an initial burst in the enzyme activity in rat prostate and epididymis homogenates has been described, which impedes the correct estimation of initial velocities with a single time point measurement. This hysteretic behavior [31, 32] was also exhibited by human prostatic 5α-reductase activity (*chapter 4*). Previously this has been described by other authors for 5α-reductase activity at pH 5.5 in the particulate fraction of human prostatic tissue [64, 72]. This latter initial burst in the time course of testosterone metabolism [64], was reportedly modified by ATP or dephosphorylating agents [65].

Several considerations have to be met to ascertain whether the observed hysteresis in enzyme kinetic characteristics is veritable [81]. Firstly, the possibility of product inhibition or substrate depletion needs to be eliminated. Both have been checked and negated in the experiments described in *chapter 2*. Secondly, the burst in enzyme activity might be attributable to enzyme inactivation during the assay, which has also been refuted in that chapter. Finally, changes in temperature, buffer and pH could cause the kinetic transients. It appears from this thesis that the sudden change in pH per se at the start of the incubation causes the initial burst in the 5α-reduction of testosterone in rat prostate, rat epididymis and human prostate. Homogenates and subcellular fractions are obtained in neutral buffers to ensure stability of enzyme activity. The hysteretic behavior of the enzyme is only observed when the assay is

performed at acidic pH, without bringing the enzyme preparation to the appropriate pH prior to the start of the incubation (*chapter 2*).

Thus, instead of a ligand induced hysteretic process [31, 32, 81], the results presented in this thesis strongly suggest that the switch from a higher to a lower activity state of the isozyme is the result of a sudden shift in pH in the microenvironment of the isozyme. These activity changes might be effectuated by the membrane environment or by certain amino acids of the enzyme, as several point mutations of the type II isozyme have also been shown to alter the acidic pH-optimum of the isozyme [51, 116]. These mutated amino acids might be sensitive to changes in pH and crucial in the tertiary structure of the protein. The results described in *chapter 2* indicate that the pH-dependent shift in enzyme activity (initial burst) differs from the variation of enzyme activity observed in a pH-profile. In the rat epididymis no higher enzymatic activity is found at pH 7.0 than at pH 5.5, although a pH-dependent initial burst in activity is exhibited by the 5α-reductase activity in this tissue (*chapter 2*).

The negative cooperativity reported for the human enzyme at acidic pH [64] differs from the non-linear Eadie-Scatchard plots of 5α-reductase activity in rat epididymis and rat and human prostate described in *chapters 3, 4 and 5*, as the experiments in these chapters were performed at pH 7.0. Furthermore, in these experiments a Tris-citrate buffer was used, whereas the negative cooperativity is reportedly not exhibited in citrate containing buffers [64]. The evidence presented in this thesis suggests that the apparent negative cooperativity encountered at neutral pH is caused by the activities of the two known isozymes of 5α-reductase. The affinity constants characteristic for the isozymes, and the correlation between isozyme ratio established from the non-linear Eadie-Scatchard plots and both the pH-dependent velocity ratio and inhibitor sensitivity, described in *chapter 5*, all strongly denote that this conclusion is valid.

7.1.3 *Acidic pH-optimum*

The peculiar acidic pH-optimum exhibited by 5α-reductase activity in certain tissues has led to the early —correct— assumption of existence of multiple isozymes of 5α-reductase [67, 79]. In literature the pH-optimum of 5α-reductase activity in rat or human tissue homogenates that express —mainly— the type II isozyme, varies from pH 5.0 to 7.0. The pH-profiles presented in this thesis have shown dissimilar optima for

rat and human 5α-reductase activities. In rat prostate and epididymis optimal activity was observed at pH 5.0, whereas the human prostatic isozyme activity was optimal at pH 5.5 (*chapter 2*). The results presented in chapter 2 indicated that at different substrate concentrations distinct pH-optima of 5α-reductase activity would be established. As the pH-optima we obtained in rat and human tissue homogenates were equivalent to the enzymes pH-optima in Vmax, thus at saturated substrate concentrations, the point raised in chapter 2 on substrate-dependency of pH-optimum is not valid as an explanation for this pH-difference between rat and human 5α-reductase. Therefore, presumably other mechanisms must also be responsible for the wide range of pH-optima obtained for 5α-reductase activity.

In this respect it is noteworthy that Thigpen and co-workers have shown that the acidic pH-optimum is probably not physiological, and that in fact both isozymes operate at neutral pH *in vivo* [109]. The authors hypothesized that after cell lysis the type II isozyme could be prone to conformational changes, e.g. due to disruption of the membrane (see *section 7.1.1*), or due to association with or dissociation of an allosteric modulator, thereby inducing an artificial acidic pH-optimum. When CHO cells transfected with the type II isozyme are gently permeabilized with digitonin, enzyme activity at pH 7.0 is maintained [109]. The binding of a second 5α-reductase subunit or protein has been proposed as a possible explanation for the absence of enzyme activity at neutral pH (indicative for type I 5α-reductase activity) in BPH extracts, despite the fact that type I specific mRNA can be found in this tissue [54]. Martin and coworkers have used this same model, i.e. binding of a second 5α-reductase subunit or protein, as a putative mechanism that would induce the initial burst they described in human prostatic tissue [22, 64].

The molecular weight of the 5α-reductase protein can be calculated as 28-29 kDa [2] and has been estimated as 26 kDa on SDS-PAGE [42]. 5α-Reductase proteins with a Mw of ~50 kDa, which might be homodimers, have been described for rat liver [69, 95] and ventral prostate [25]. Therefore, dimerization might be a mechanism to influence 5α-reductase isozyme activities, either effectuating the acidic pH-optimum [109], the reported lack of type I enzymatic activity in the human prostate [54], or the initial burst at acidic pH [22, 64]. Remarkably, allosteric enzymes are often dimeric. If dimerization indeed occurs after homogenization, the dimer might exhibit allosteric kinetics. Binding of substrate to one binding domain could subsequently influence binding to the other (through site-site interactions), leading to positive or negative

cooperativity [80]. Indeed, negative cooperativity has been reported in the human prostatic isozyme at pH 5.5 [64], thus at the peculiar acidic pH-optimum.

Finally, in human prostatic epithelium the enzyme prostatic acid phosphatase (PAP) is highly active [105]. This enzyme is most active at pH 5.0 to 5.5 [98]. 5α-Reductase activity is reportedly influenced by (de)phosphorylation by PAP [65]. This might also be a cause for the acidic pH-optimum observed after homogenization. The experiments described in this thesis, however, could not corroborate an effect of the phosphate donor ATP on enzyme activity (*chapter 2*).

Altogether, we agree with Thigpen and coworkers that the acidic pH-optimum of the type II isozyme is most probably non-physiological and likely to be a mere consequence of cell lysis. This prompted us to perform all further experiments on 5α-reductase reported in this thesis at neutral pH (*chapters 3, 4 and 5*). As delineated in *chapter 2*, this choice of experimental pH will have a major impact on the results subsequently obtained for 5α-reductase kinetic characteristics.

7.1.4 *Quantification of enzyme activity in vitro and its relation to in vivo conditions*

The aim of this thesis was to study some of the problems associated with the classification and quantification of 5α-reductase activity. As mentioned in the introduction, some of the discrepancies between previous reports might be attributed to the different methods of quantification. Isozymes can be quantified on the mRNA, protein and on the enzymatic activity level. If measured on the activity level, isozymes can be compared at their optimal pH or at neutral pH.

We adhered to the supposition to measure both isozymes at neutral pH. To be able to distinguish between two isozymes at the same pH, we applied a wide range of testosterone concentrations and plotted the estimated initial velocities according to Eadie-Scatchard. As can be seen in figures 3.2, 3.3 and 4.2, a smaller testosterone concentration range could result in a straight line when plotting according to Lineweaver-Burke or Eadie-Scatchard. Plotting the wide range of substrate concentrations we applied according to Lineweaver-Burke could also results in an oversight of the presence of two isozymes. We quantified enzymatic activity both by their Vmax, and by their efficiency ratio Vmax/Km. The Vmax of an enzyme describes the maximum velocity the enzyme can achieve at high, saturating substrate

concentrations. However, as the testosterone tissular concentrations *in vivo* are much lower than the Km of 5α-reductase, the efficiency ratio Vmax/Km might be a better quantification parameter. According to this latter parameter, results presented in this thesis suggest that the type I isozyme hardly contributes to DHT formation in rat epididymis and human prostate, at least *in vitro*. On the other hand, the *in vivo* situation might still be quite different. In figure 7.1 several speculative mechanisms are depicted whereby the low affinity of the type I 5α-reductase isozyme does not preclude DHT formation by this subtype at physiological substrate concentrations.

The first mechanisms involve active transport of testosterone into the cell. This can be effectuated by a Sex Hormone Binding Globulin (SHBG) or Androgen Binding Protein

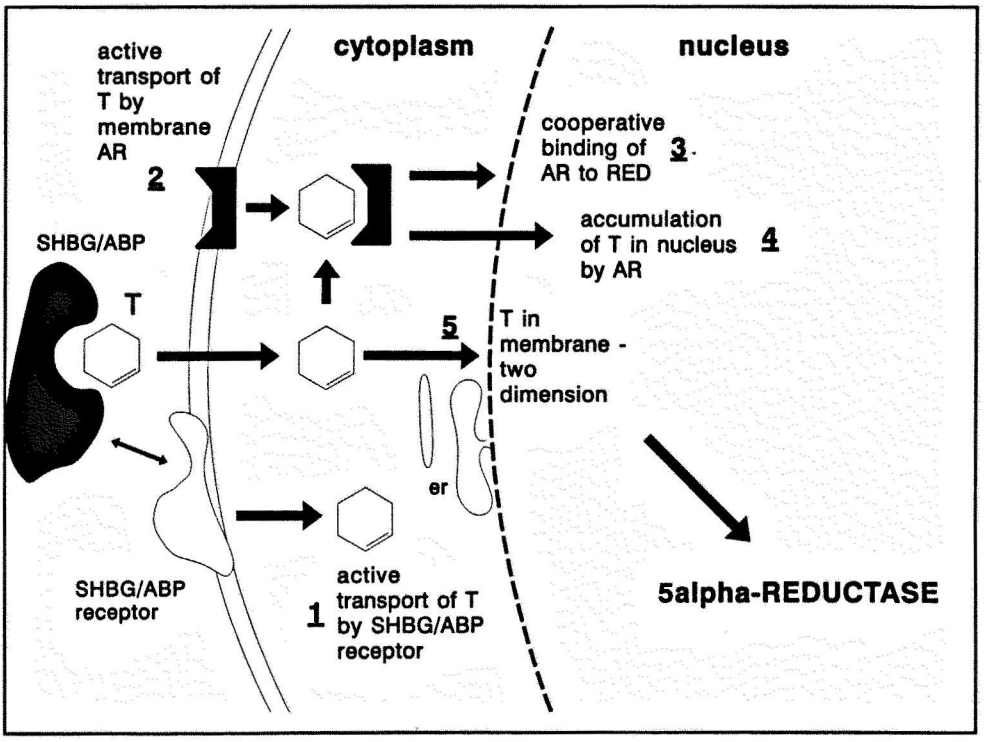

Figure 7.1: several speculative mechanisms whereby the substrate concentration could be raised and the contribution of 5α-reductase type I to DHT formation augmented. 1: The SHBG/ABP receptor or 2: membrane-associated androgen receptor (AR) actively transports testosterone (T) into the cell, thus raising the intracellular T concentration. 3: The AR binds to 5α-reductase, thereby delivering substrate directly to the enzyme. 4: The AR accumulates T in the nucleus, thereby raising the substrate concentration in the microenvironment of the enzyme. 5: T enters the membrane (endoplasmatic reticulum (er) or nuclear envelope), thereby limiting the compartment of interaction to two dimensions. For references, see text.

(ABP) receptor (*figure 7.1,* 1), either of which has been detected on rat epididymis epithelial cells [27, 39] and in human prostate [47], and has been shown to be internalized in epididymal cells [34, 38]. The metabolism of testosterone by human prostatic slices is reportedly different when SHBG instead of albumin is added to the medium [75]. Chronic infusion of SHBG in rats, although leading to a marked decrease in free testosterone, does not influence testosterone action in these animals [21]. The SHBG receptors are only expressed in androgen-dependent tissues [28]. All this suggests a role for the SHBG receptor in active cellular androgen uptake, additional to diffusion. However, some reports indicate that the SHBG/ABP receptor is also involved in intracellular signalling [88]. Binding proteins with characteristics similar to the classical androgen receptor have been detected on rat liver and prostate cell membranes [58], which might also mediate active androgen uptake in these tissues (*figure 7.1,* 2). In this respect, the report on the cooperative binding of the androgen receptor with 5α-reductase [56] is also interesting (*figure 7.1,* 3). This might represent a mechanism of active transport of substrate directly to the enzyme. Furthermore, the internalization of the testosterone-androgen receptor complex into the nucleus [37, 85] would lead to an active transport of substrate to the microenvironment of the enzyme (*figure 7.1,* 4). This compartmentalization will lead to a higher reaction rate. Our results on the nuclear localization of the type I isozyme in the rat prostate (*chapter 5*) are in line with such a hypothesis. The final mechanism whereby the reaction rate of the enzyme can be increased, is by restricting the diffusion of reactants to the two dimensions of a membrane (*figure 7.1,* 5) [1, 6]. The highly hydrophobic 5α-reductase isozymes are unequivocally membrane-bound [15, 26, 30, 45, 57, 67, 70, 77, 78, 94]. As discussed in section 7.1, the hydrophobicity of the steroid binding domain of 5α-reductase and the correlation between substrate polarity and apparent affinity for the enzyme suggest the substrate must enter the membrane in order to bind to 5α-reductase. Therefore, the mechanism whereby the substrate concentration is relatively raised due to the restriction of the compartment of substrate-enzyme interaction to the two dimensions of a membrane (either the nuclear envelope or the endoplasmatic reticulum) seems conceivable. The aforementioned mechanisms would subsequently render the premise $[S] << Km$, and thus the justification of the use of the Vmax/Km ratio [59, 112] as a method of quantification invalid. Thereby, the low affinity of the type I isozyme for testosterone would be overcome and this subtype would be able to significantly contribute to DHT formation.

Furthermore, as mentioned earlier, enzyme quantification is usually performed at optimal conditions, including optimal substrate and cofactor concentrations. *In vivo*, the situation may be quite different. For instance, citrate metabolism generates ATP, which reportedly augments 5α-reductase activity [65], but also NAD(P)H. Citrate is found in prostatic epithelium and is in fact a major secretory component of the prostatic epithelium [16]. Citrate concentrations are low in prostatic carcinoma and elevated in BPH [16, 17]. The extensive formation of citrate in the human prostate thus most probably influences the *in vivo* concentrations of cofactors in a cell. The intracellular concentrations of NAD(P)(H) has a tremendous influence on DHT formation and degradation as reported in *chapter 6* for HSOR activities. For a correct interpretation of data obtained *in vitro*, these considerations should be kept in mind.

7.2 POSSIBLE INVOLVEMENT OF TYPE I 5α-REDUCTASE ACTIVITY IN HYPERPLASIA OF THE HUMAN PROSTATE

Benign Prostatic Hyperplasia (BPH) develops after the fourth decade of life and leads to urinary obstruction symptoms in 30-40% of all men after sixty years of age [23, 44]. The incidence of histopathological BPH is 50% at sixty and almost 100% at eighty years of age. Eventually, one in four men will have to be treated for symptoms of BPH [4]. This usually required —until recently— transurethral resection of the prostate (TURP) to relieve symptoms. The morbidity of this procedure is 15 to 18% [44]. Therefore a substantial amount of research has focused on the pharmacological treatment of BPH. For some time now it has been appreciated that growth of the prostate is controlled by androgens and it has been proposed that an elevation of tissular DHT concentration plays a role in the pathogenesis of BPH [101]. As 5α-reductase is the enzyme responsible for the formation of DHT, research has focused on the pharmacological attenuation of 5α-reductase activity for the treatment of patients with BPH. Of the two isozymes of 5α-reductase, it was the type II isoform that was implicated in the pathogenesis of BPH as this subtype is mainly expressed in androgen target tissues [82] and patients with type II 5α-reductase deficiency (male pseudohermaphroditism) have atrophic prostates [29, 50]. Therefore, a type II 5α-reductase-specific inhibitor, finasteride (Proscar®), has been developed for the treatment of patients with BPH [35]. This inhibitor, however, only leads to reduction of urinary obstruction symptoms in a subset of patients [83, 106].

Recently, mRNA of the type I isozyme has been detected in both normal prostatic and

BPH tissue [9, 71], although this could not be confirmed [111] (*section 1.7, tables 1.1 and 1.2*). The results reported in *chapter 4* of this thesis provide, for the first time, evidence for the presence of type I 5α-reductase in the human hyperplastic prostate on the enzymatic activity level. A 6-times higher Vmax is found for the type I than for type II 5α-reductase activity in prostate homogenates. Type I 5α-reductase, expressed in the liver of both rat and man, was originally considered primarily to participate in the metabolism and excretion of steroid hormones like testosterone (catabolic enzyme) [82]. The presence of type I 5α-reductase activity in the human prostate, reported in this thesis, indicates that this subtype might also play an anabolic role. This section will therefore address the possibility of type I 5α-reductase involvement in growth and maintenance of the human prostate, and, in addition to type II, in the pathogenesis of BPH.

In order to discern the role of type I 5α-reductase in the human prostate, its specific localization has to be taken into account (*table 1.2*). The cell-type specific localization of the 5α-reductase subtypes has been investigated by several authors. On the *mRNA level*, detection of specific mRNA has as yet not been extended to cell-type specific localization [9]. However, the epithelium-derived prostatic cell-line DU-145 expresses type I specific mRNA [22], suggesting the type I would be localized in epithelium of the human prostate. Type I 5α-reductase *immunoreactivity* has not been found in the human prostate [102, 103], although a rat type I antiserum did bind to a protein in nuclei of both stromal and epithelial cells of the human prostate [42]. Antibodies against the type II isoform have located 5α-reductase in the basal epithelial cells of the human prostate [24], but also in stromal cells of this tissue [102, 103].

On the *activity level*, previous reports on isozyme specific expression in the human prostate have not been irrefutable. Several differences between stromal and epithelial 5α-reductase in the human prostate have been described that might indicate a distinct localization of the isozymes. The affinity constants for testosterone vary, being higher in stroma (23 to 230 nM) than in epithelial cells (11 to 90 nM) [12, 49, 59, 90, 114], suggesting, although both are in the range of the type II isozyme, a more stromal localization of the type I isozyme and a predominantly epithelial localization of the type II. The 5α-reductase activity found in epithelium is more sensitive to finasteride (Ki = 7 ± 3 nM, IC_{50} = 38 nM) than stromal activity (Ki = 31 ± 3 nM, IC_{50} = 112 nM) [61, 114], again suggesting a more stromal localization of the type I isozyme. The more acidic pH-optimum of epithelial 5α-reductase (pH 6.5) than that in stroma (pH

7.4) [49] seems to corroborate this finding. Furthermore, zinc, which has a biphasic effect on prostatic 5α-reductase activity [36, 40, 48, 63, 113] exerts a stronger inhibitory effect on stromal 5α-reductase than on epithelial enzymatic activity [49]. As only the type I isozyme is inhibited by zinc [107], a more stromal localization of the type I isozyme activity is again suggested. The aforementioned isozyme characteristics do not unequivocally demonstrate a dissimilar cell-type specific localization of the isozymes. This, however, might be attributed to either the incomplete separation of stromal and epithelial prostate fractions and/or to the use of a substrate range inappropriate for detecting type I 5α-reductase isozyme activity. Summarizing, these data from literature seem to corroborate our finding of the presence of type I 5α-reductase enzymatic activity in the human prostate. Furthermore, these data suggest a predominantly stromal localization of type I 5α-reductase activity, and a more epithelial localization of type II activity.

Whether the type I isozyme is capable of generating significant amounts of DHT, considering its low affinity for T, has been discussed in *section 7.1.4*. Furthermore, reports comparing epithelial and stromal cells of human prostate in 5α-reductase capacity have shown either an equal 5α-reductase activity [5, 41, 96], or a higher amount of activity (Vmax) in stroma [12, 18, 60, 61]. As stromal 5α-reduction of T seems to be effectuated preferentially by the type I isozyme (*see above*), this would suggest this subtype is indeed capable of generating appreciable amounts of DHT. Remarkably, in rat prostate, epithelial 5α-reductase is more active than stromal enzyme activity [86, 87]. In this tissue, contrary to the situation in the human prostate, the type I isozyme is located in the epithelium, as established by mRNA measurements and immunohistochemistry [7]. Thus, again, the cell-type that expresses type I 5α-reductase seems most active in generating DHT.

DHT, formed by 5α-reductase, will bind to the androgen receptor (AR) to exert androgenic effects [14, 84, 85]. Therefore, to ascertain the role of the 5α-reductase isozymes, the cell-type localization of the AR is vital. During embryogenesis, a functional AR in stroma, but not in epithelium, is essential for normal prostatic development [19, 20]. Indeed, AR is found prenatally in stroma, and not in epithelium of rat prostate [108]. In contrast, in adulthood the AR is expressed mainly in glandular epithelial cells [53], but also in stroma of rat [13, 55, 60] and human prostate [62, 104]. Remarkably, castration or 5α-reductase inhibition only leads to atrophy and apoptosis of epithelial cells [89, 91, 99]. Thus the stromal cells do not require

androgen for growth and maintenance. Currently it is presumed that DHT binds to the AR in stroma, thereby instructing this cell-type to produce paracrine factors that induce epithelial morphogenesis [20, 52] or that DHT produced in stroma binds to the epithelial AR [106]. Either mechanism would suggest a role for the type I isozyme in DHT formation in stromal cells to influence epithelial cell morphogenesis.

Finally, the human prostate can be subdivided in several zones [73, 74]. Prostate cancer mainly originates from glandular epithelial cells [52] in the peripheral zone of the human prostate [106]. BPH is initially confined to either the transition or periurethral zone [73, 106]. The BPH nodules that arise in the transition zone tend to be more epithelial, whereas nodules in the periurethral zone initially are stromal [106]. Thus, it seems possible that BPH is a heterogeneous disease, originating either from stromal cells or from epithelial cells. As the type II inhibitor finasteride only leads to relieve of urinary obstruction symptoms in a subset of patients [83, 106], one has to consider the possibility that two forms of hyperplastic growth exist, one in which the type II 5α-reductase is involved, and another which might be associated with altered type I activity.

Prostate biopsies from patients with symptomatic BPH contain more stromal elements than those of patients with BPH but without urinary obstruction symptoms [97]. There is a lack of correlation between prostate size and urinary obstruction symptoms in patients with BPH. This could depend on the type of hyperplastic growth, as stroma mainly consists of smooth muscle cells. Growth of these contractile smooth muscle cells could more easily lead to obstruction symptoms than would epithelial hyperplastic growth. Type I 5α-reductase enzymatic activity in the human prostate described in this thesis seems to significantly contribute to the amplification of the anabolic effects of testosterone in stromal cells. Establishment of the role of type I 5α-reductase in BPH is thus essential to evaluate the necessity of (supplementary) type I inhibition in the treatment of patients with this neoplasia. Already a specific human type I inhibitor (LY191704) is available [43], while more type I inhibitors are being developed [33, 68]. In light of the results presented in this thesis, and the reports discussed in this paragraph, it seems attractive to test these inhibitors, alone or in combination with type II inhibitors, for their efficacy in treatment of patients with BPH.

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CHAPTER 8
SUMMARY AND CONCLUSIONS

This thesis focuses on the measurement of the enzymatic activities of the two known isozymes of 5α-reductase, type I and type II. The product of 5α-reduction of testosterone, DHT, has been implicated in a wide variety of physiological mechanisms and in the pathogenesis of several diseases and disorders as benign prostatic hyperplasia (BPH), and possibly prostatic carcinoma, acne, hirsutism and alopecia. Measurements of the enzymatic activities of 5α-reductase are hampered by the kinetic peculiarities of mainly the type II isozyme and by the lack of an assay that can distinguish between both isozymes. The aim of the experiments described in this thesis is to set up a valid assay for both isozyme activities, and to subsequently quantify their activities in rat prostate and epididymis and in the human prostate. The intracellular localization of their activities are assessed in the rat prostate to get more insight into the role of the 5α-reductase isozymes. Finally, enzymes that degrade DHT (as HSOR) are studied, to get an impression of the degradation, in addition to the formation of this steroid hormone.

In *chapter 2*, several of the kinetic peculiarities of the isozymes are studied in homogenates of the rat prostate and epididymis. The enzymatic activity of both type I and type II 5α-reductase appears unstable during homogenization, which impedes correct kinetic evaluation of the isozymes. Prebinding the enzyme before homogenization with its cofactor, NADPH, preserves much of the activity. Furthermore, at acidic pH, the time course of 5α-reduction of testosterone is non-linear in these tissues, showing an initial burst, which hampers the estimation of initial velocities in a single time point measurement. This initial burst is shown to result from the sudden shift in pH in the microenvironment of the enzyme at the start of the incubation. The homogenate is prepared in a buffer of neutral pH, whereas the assay is performed at acidic pH. When the enzyme preparation is brought to acidic pH beforehand, the initial burst in activity is abolished. Subsequently, 5α-reductase activity is studied over a pH-range of 4.5 to 8.0. Especially at acidic pH, the kinetic characteristics Vmax (maximum velocity, an indication of the amount of enzyme) and Km (apparent affinity constant of the enzyme for its substrate) vary manifold. Minor changes in experimental pH will have a substantial influence on kinetic parameters and thereby on enzyme velocities. This could explain the variation in these

parameters reported in literature and discrepancies in described effects on enzymatic activities.

Although the type II 5*α*-reductase isozyme is optimally active at acidic pH *in vitro*, previous work of other authors have shown that both isozymes probably operate at neutral pH *in vivo*. In *chapter 3*, 5*α*-reductase enzymatic activities are studied at neutral pH in the rat prostate and epididymis. Eadie-Scatchard plots of initial velocities against a wide range of testosterone concentrations are non-linear in these tissues. These non-linear plots can indicate cooperativity in the enzymatic reaction, or the expression of isozymes. So far, the presence of two isozymes has been described on the mRNA-, protein- and enzymatic activity level in rat prostate. In the rat epididymis, the type I isozyme has only been found on the mRNA and protein level. Applying the assay established and described in chapter 3, both isozyme activities are quantified at neutral pH. In the rat prostate, a 50-times higher Vmax is found for the type I isozyme than for the type II at neutral pH. In the rat epididymis type I 5*α*-reductase activity is established for the first time. The maximum velocities of both isozymes are approximately equal in this tissue.
As the type I isozyme has a relatively low affinity for steroid hormones, other authors have proposed the Vmax/Km ratio to quantify enzymatic activities. This ratio is a measure for enzymatic activity at low substrate concentrations. Using the Vmax/Km ratio as method of enzymatic activity quantification, the type I isozyme contributes to 25% of the total potential *in vivo* activity in the rat prostate. The Vmax/Km ratio of the type I isozyme in the rat epididymis suggests that this subtype hardly contributes to DHT formation in this tissue. The highly segmental expression of the type I 5*α*-reductase isozyme in the rat epididymis, however, could still implicate this subtype in DHT formation.

In *chapter 4*, the same assay is used to quantify isozyme activities in homogenates of the human hyperplastic prostate. The type II isozyme is implicated in the pathogenesis of prostatic hyperplasia, which eventually necessitates transurethral resection in 25 % of all men. Furthermore, type II inhibitors have been developed for the pharmacological treatment of BPH. Verifying the presence of type I 5*α*-reductase in this tissue might be important to establish optimal pharmacological treatment of patients with BPH. Results described in this chapter indicate, for the first time, type I 5*α*-reductase isozyme activity in the human prostate. The maximum velocity of this type I activity is 6-times higher than that of the type II isozyme. However, if isozyme

activities are quantified by their Vmax/Km ratio, this method would indicate that the type I hardly contributes to DHT formation in the human prostate. Future research will have to focus on the validity of Vmax or Vmax/Km ratio as a method of enzyme activity quantification.

So far, the type I 5α-reductase isozyme has been considered to exert *catabolic* effects, being mainly engaged in the degradation of steroid hormones, whereas the type II was considered *anabolic*, serving to amplify the androgenic effects of testosterone. To get more insight into the role of the isozymes in androgenic action, the subcellular distribution of the 5α-reductase isozyme activities is studied in *chapter 5*. Surprisingly, the type I isozyme appears predominantly nuclear bound, whereas the type II isozyme is mainly detected in the microsomal fraction. This would strongly suggest that the type I isozyme is implicated in the amplification of androgenic action of testosterone in this tissue. The proposed catabolic role of this isozyme does therefore not seem to concur with its subcellular localization and ample expression in the rat prostate.

The tissular DHT concentration is the result of a balance between several enzymatic activities. The degradational product Adiol can also be back-oxidized to DHT. To get more insight into the formation and degradation of DHT *in vivo*, the metabolism of DHT is studied in homogenates of rat prostate and epididymis (*chapter 6*). The 3α-HSOR-mediated reduction to Adiol is the major degradational route of DHT *in vitro*. At cofactor concentrations reportedly present *in vivo*, i.e. [NADPH] $>>$ [$NADP^+$] and [NAD^+] $>>$ [NADH], tissular DHT concentrations will be maintained in both these tissues. The established enzymatic activities suggest a potentially important function of the cofactors in the regulation of tissular DHT concentration.

The results described in this thesis shed more light on the kinetic peculiarities of the isozymes of 5α-reductase. The method described for the measurement of isozyme activities at neutral pH is capable of detecting both isozymes in androgen target tissues, i.e. the rat prostate, epididymis and the human prostate. The expression of type I 5α-reductase in the human prostate, described in this thesis, warrants further research into the role of this isozyme in the pathogenesis of benign prostatic hyperplasia.

CHAPTER 9
SAMENVATTING EN CONCLUSIES

In dit proefschrift staat de meting van de enzymatische activiteit van de twee bekende isozymen van 5α-reductase, type I en type II, centraal. Het produkt van 5α-reductie van testosteron, DHT, wordt van belang geacht bij een veelvoud aan fysiologische processen, maar ook bij het ontstaan van verschillende ziektebeelden en aandoeningen, als prostaat-hyperplasie en mogelijk ook prostaat-carcinoom, acne, hirsutisme en alopecia. Experimenten waarin men de enzymatische activiteit van 5α-reductase wil meten worden gehinderd door de bijzondere kinetische eigenschappen van voornamelijk het type II isozym, maar vooral ook door het feit dat er tot nu toe geen enzymatische activiteits-metingen beschikbaar zijn waarmee beide isozymen kunnen worden onderscheiden. Het doel van het onderzoek beschreven in dit proefschrift is een valide meting op te zetten voor beide 5α-reductase isozym-activiteiten, waarmee hun activiteiten in de prostaat en de epididymis van de rat en in de humane prostaat kunnen worden gekwantificeerd. Om meer duidelijkheid te krijgen over de functies van de 5α-reductase isozymen, is ook hun intracellulaire lokalisatie bepaald in de prostaat van de rat. Tenslotte zijn de enzymen die DHT afbreken (HSOR) bestudeerd om, naast over de aanmaak, ook over de afbraak van dit hormoon een indruk te krijgen.

In *hoofdstuk 2* zijn enkele van de kinetische eigenschappen van de isozymen bestudeerd in homogenaten van de prostaat en de epididymis van de rat. De enzymatische activiteit van zowel het type I als het type II 5α-reductase blijkt tijdens homogeniseren van het te onderzoeken weefsel niet stabiel te zijn, hetgeen een correcte evaluatie van de verkregen kinetische gegevens onmogelijk maakt. Uit ons onderzoek is gebleken dat na toevoeging van de cofactor NADPH vóór het homogeniseren echter een groot deel van de enzymatische activiteit behouden blijft. Bovendien blijkt dat bij een lage pH de 5α-reductie van testosteron niet lineair met de tijd is in deze weefsels. In het begin van de meting vindt een bijzonder snelle enzymatische omzetting plaats, gevolgd door een periode met minder enzymatische activiteit. Het bepalen van enzymatische snelheden door middel van een enkel meetpunt is daardoor niet valide. De oorzaak blijkt te zijn gelegen in een plotselinge verandering van de pH bij de start van de enzymatische meting. Het weefsel-homogenaat wordt namelijk bereid in een buffer met neutrale pH, terwijl de reactie

wordt gemeten bij lage pH. Door het enzym preparaat vóór de start van de incubatie op gewenste pH te brengen, wordt de 5α-reductie van testosteron wel lineair met de tijd. Vervolgens is de 5α-reductase activiteit gemeten over een pH bereik van 4.5 tot 8.0. Vooral bij lage pH blijken de kinetische parameters Vmax (maximale snelheid, een maat voor de hoeveelheid enzym) en Km (de affiniteits-constante van het enzym voor een substraat) sterk te variëren. Kleine experimentele pH-verschillen hebben grote verschillen in kinetische parameters en dus ook in enzymatische activiteit tot gevolg. Dit zou een mogelijke verklaring kunnen zijn voor de grote variatie in deze parameters gerapporteerd in de literatuur en voor de discrepanties in beschreven effecten op enzym activiteiten.

Hoewel het type II isozym *in vitro* een optimale activiteit vertoont bij lage pH, blijkt uit in de literatuur beschreven onderzoek dat beide isozymen *in vivo* waarschijnlijk toch bij neutrale pH werken. In *hoofdstuk 3* is de activiteit van beide 5α-reductase isozymen in de prostaat en epididymis van de rat bestudeerd bij pH 7.0. In deze weefsels blijken zogenaamde Eadie-Scatchard plots, waarbij de activiteit uitgezet wordt tegen een uitgebreide reeks van testosteron concentraties, niet lineair te zijn. Een niet-lineaire Eadie-Scatchard plot duidt ofwel op coöperativiteit in de enzymatische reactie, of op de aanwezigheid van isozymen. In de ratte-prostaat zijn twee isozymen beschreven op zowel mRNA, eiwit als op enzymatisch activiteits niveau. In de ratte-epididymis echter is het type I alleen op mRNA en eiwit niveau aangetoond. Uit de bovengenoemde Eadie-Scatchard plots blijkt dat het type I, bij neutrale pH, een 50 maal hogere Vmax heeft dan het type II in de prostaat. In de epididymis wordt met deze methode voor het eerst type I enzymatische activiteit aangetoond. In dit weefsel is de Vmax voor beide isozymen gelijk.
Omdat het type I 5α-reductase een lage affiniteit heeft voor steroïd-hormonen, is eerder door andere auteurs de Vmax/Km ratio voorgesteld om enzymatische activiteiten te kwantificeren. Deze ratio is per definitie een maat voor enzymatische activiteit bij relatief lage substraat concentraties. De Vmax/Km ratio geeft aan dat het type I voor 25% verantwoordelijk is voor de vorming van DHT in de prostaat van de rat, maar nauwelijks bijdraagt aan de vorming van DHT in de epididymis. Echter, de sterk gelokaliseerde expressie van dit subtype in een specifiek segment van de ratte-epididymis zou wellicht toch kunnen leiden tot een significante bijdrage van dit type I 5α-reductase aan de omzetting van testosteron naar DHT.

In *hoofdstuk 4* is van de ontwikkelde meting gebruik gemaakt om de enzymatische

activiteit in homogenaten van de humane hyperplastische prostaat op isozym niveau te bepalen. Type II 5α-reductase speelt mogelijk een rol bij het ontstaan van hyperplasie van de prostaat, welke bij 25 % van alle mannen een operatieve behandeling noodzakelijk maakt. Daarnaast zijn type II 5α-reductase remmers ontwikkeld om deze hyperplasie medicamenteus te kunnen behandelen. Tot nu toe is aanwezigheid van het type I enzym in dit weefsel alleen op mRNA niveau beschreven. Kennis van de eventuele aanwezigheid van type I 5α-reductase kan van belang zijn bij het vaststellen van een optimale medicamenteuze behandeling van prostaat hyperplasie. In dit proefschrift wordt de aanwezigheid van type I 5α-reductase enzymatische activiteit in de humane prostaat voor het eerst aangetoond. De Vmax van type I 5α-reductase in dit weefsel is zelfs 6 maal zo hoog als dat van het type II. Wanneer de isozymen worden gekwantificeerd volgens hun Vmax/Km ratio, dan lijkt type I nauwelijks bij te dragen aan vorming van DHT in de humane prostaat. Verder onderzoek zal moeten aangeven of de Vmax of de Vmax/Km ratio een valide methode voor de kwantificering van enzymatische activiteiten is.

Tot nu toe werd de functie van het type I 5α-reductase isozym als *catabool* beschouwd, betrokken bij de afbraak van steroïden, en dat van het type II isozym als *anabool*, betrokken bij het versterken van de androgene effecten van testosteron. Om een beter inzicht te verkrijgen in de functies van beide 5α-reductase isozymen m.b.t. androgene effecten, is in *hoofdstuk 5* de subcellulaire lokalisatie van isozym activiteiten in de ratte-prostaat bepaald. Opvallend is dat het type I 5α-reductase voornamelijk in de kern gelokaliseerd blijkt te zijn, terwijl het type II meer microsomaal wordt gevonden. Dit zou betekenen dat het type I wel degelijk een rol speelt bij de versterking van de androgene effecten van testosteron op dit weefsel. De veronderstelde catabole functie van dit isozym lijkt derhalve niet overeen te komen met zijn subcellulaire lokalisatie en aanzienlijke expressie in de prostaat van de rat.

De weefsel-DHT concentratie wordt bepaald door de balans tussen diverse enzymatische activiteiten. Het belangrijkste afbraak produkt van DHT, Adiol kan ook weer terug worden geoxideerd tot DHT. Om een betere indruk te krijgen van de vorming en afbraak van DHT *in vivo* is het metabolisme van DHT bestudeerd in homogenaten van ratte-prostaat en -epididymis (*hoofdstuk 6*). De door het enzym 3α-HSOR gemediëerde omzetting tot Adiol is de belangrijkste route van DHT afbraak, althans zeker *in vitro*. Bij *in vivo* geldende cofactor concentraties, waarbij [NADPH] >> [$NADP^+$] en [NAD^+] >> [NADH], zullen de gevonden enzym-activiteiten

resulteren in een netto aanmaak van DHT. De gemeten enzymatische activiteiten duiden op een mogelijk belangrijke functie van de betrokken cofactoren in de regulatie van de weefsel DHT-concentratie.

De resultaten beschreven in dit proefschrift geven meer duidelijkheid over de kinetische eigenschappen van de 5α-reductase isozymen. De beschreven methode voor de meting van isozym-activiteiten bij neutrale pH, stelt ons in staat om beide isozymen te detecteren in androgeen-doelwitorganen, namelijk de ratte-prostaat, -epididymis en humane prostaat. Gezien de aanwezigheid van type I 5α-reductase in de humane prostaat, zoals beschreven in dit proefschrift, dient de rol van dit type 5α-reductase bij het ontstaan van prostaat-hyperplasie verder te worden onderzocht.

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DANKWOORD

Op de eerste plaats wil ik hier mijn ouders, broer en zus bedanken. Hun steun tijdens en na mijn studie was onontbeerlijk voor het ontstaan van hetgeen nu voor jullie ligt. Hun voortdurende belangstelling, ondanks de moeilijke tijden gedurende de afronding van dit proefschrift, is mij zeer dierbaar geweest.

Verder ben ik Prof. Smals, Prof. Benraad en Fred Sweep zeer erkentelijk. Jullie hebben mij alle vrijheid gegeven om van mijn fouten te leren, welke kans ik dan ook met twee handen aangegrepen heb. Ondanks alle opstart- en vervolgproblemen bleven jullie mij steunen. Ook Dick, die mij de eerste paar maanden onder zijn hoede nam en mij de radio-immunoassay leerde, en Leon en Harry voor het 'HPLC-onderwijs' wil ik hierbij bedanken. Harry, ondanks jouw keuze van muziek was je een prima collega en huisgenoot.

Het Laboratorium voor Endocrinologie en Voortplanting wordt met al zijn werknemers bij deze bedankt voor hun hulp, steun en gezelligheid. Vooral voor dit laatste natuurlijk 'het cafe': Angeline, Carla, Jan, Jantje, Jolanda, Rob, Sjaak. Mijn woordgebruik is door jullie in de afgelopen jaren een stuk kleurrijker geworden. Voor 'materiele' ondersteuning bij het onderzoek wil ik hier Doorlene, Ernst, Jan en Joop bedanken, en voor secretariele ondersteuning Marieke.

Leden van Phocas, met name Arthur, Ashwin, Ben, Cantor, Daan, Durk, Mark, Miel, Quinten, Willem en Wim, worden hierbij bedankt voor hun vriendschap. Door jullie was de overgang van student naar werknemer een stuk minder schokkend. Hans en Quinten wil ik bedanken voor hun werk als paranimf, hetgeen zij ongetwijfeld met veel inzet hebben gedaan.

En Loes: zonder te overdrijven kan ik gerust stellen dat zonder jou ik dit niet voor mekaar had gekregen. Ongelooflijk, nooit werd jij moe van mijn gepraat over roeien en steroïden.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 25 april 1967 geboren te Utrecht. Het Gymnasium-β werd met goed gevolg afgesloten aan het scholengemeenschap Jeanne-d'Arc te Maastricht in 1985. Datzelfde jaar begon hij met de studie Biologie in zijn geboortestad. In 1986 behaalde hij (cum laude) zijn propadeuse-diploma. Zijn hoofdvakstage heeft hij in 1988-1989 gedaan bij Dr. J. Lambert van de vakgroep Endocrinologie van de faculteit Biologie (Hoofd: Prof. Dr. P.G.W.J. van Oort). Daarbij deed hij onderzoek naar het steroïd-metabolisme in de gonaden van de Europese paling. Ook werd hier, d.m.v. GC-MS, steroïden in de hypofyse van de Afrikaanse meerval gemeten. Zijn bijvakstage werd gevolgd in 1989-1990 bij de vakgroep Biomedische Farmacologie van de faculteit Farmacie onder leiding van Mw. Drs. D.M.J. Veenstra en Dr. K.J.H. van Buuren (Hoofd: Prof. Dr. F.P. Nijkamp). Hierbij werd onderzoek verricht naar de farmacologische subtypering van α_1-adrenerge receptoren op de cavia-aorta. In deze tijd heeft hij, op dezelfde afdeling, ook meegewerkt aan een onderzoek onder leiding van Dr. G. Folkerts naar de door het parainfluenza-3 virus geïnduceerde bronchiale hyperreactiviteit bij de cavia. De afstudeerscriptie had als onderwerp *'Het Postreceptor-mechanisme van de α_1-adrenerge Receptor-subtypen'*. Zijn doctoraal-diploma behaalde hij in december 1990.

In december 1990 werd hij aangesteld als assistent-in-opleiding bij de vakgroep Endocriene Ziekten van de afdeling Inwendige Ziekten van het St. Radboud Ziekenhuis te Nijmegen (Hoofd: Prof. Dr. P.W.C. Kloppenborg). Onder leiding van Prof. Dr. A.G.H. Smals en Prof. Dr. Th.J. Benraad werd het in dit proefschrift beschreven onderzoek verricht.

The two isozymes of steroid 5α–reductase mediate in the conversion of testosterone to the potent androgen dihydrotestosterone. Distortion of 5α–reductase activity is considered implicated in the pathogenesis of a variety of androgen–dependent diseases like Benign Prostatic Hyperplasia.

This thesis addresses the assessment of 5α–reductase isozyme activities in rat and human androgen target tissues. The obtained results warrant a reevaluation of the proposed dissimilar roles of the 5α–reductase isozymes type I and II in androgen metabolism as catabolic and anabolic respectively.

The work presented in this thesis has been performed in the Department of Medicine, Division of Endocrinology and in the Laboratory of Endocrinology and Reproduction of the Academic Hospital Nijmegen St.Radboud, The Netherlands.